A Fragment of Triosephosphate Isomerase Competes with the Vasoactive Intestinal Polypeptide (VIP) for Binding to the VIP Receptor

Guro Gafvelin, Tomas Bergman, Mats Andersson, Bengt Persson, Hans Jörnvall and Viktor Mutt

Departments of Biochemistry II and Chemistry I, Karolinska Institute, Box 60400, S-104 01 Stockholm, Sweden

Gafvelin, G., Bergman, T., Andersson, M., Persson, B., Jörnvall, H. and Mutt, V., 1991. A Fragment of Triosephosphate Isomerase Competes with the Vasoactive Intestinal Polypeptide (VIP) for Binding to the VIP Receptor. – Acta Chem. Scand. 45: 63–67.

A 28-residue *N*-terminal fragment of triosephosphate isomerase, TIM(1–28), has been purified from porcine upper intestine. It competes with VIP for binding to the VIP receptor on rat liver plasma membranes with an IC_{50} value of 2.8 mM, about 1000 times higher than that for VIP binding to the same membranes. Except for a single positional identity and the number of amino acid residues, the amino acid sequences of TIM(1–28) and VIP are unrelated as regards primary structure. However, the ability to bind to the same receptor site may indicate common three-dimensional structural properties.

Vasoactive intestinal polypeptide (VIP) is a neurohormonal peptide belonging to the glucagon–secretin family of peptides. It was originally isolated from porcine intestine[1] but VIP-like immunoreactivity has since been found both in the central and the peripheral nervous system.[2] All known mammalian VIPs consist of 28 amino acid residues and have a *C*-terminal asparagine amide structure. Although the primary structure of VIP has been known for quite a long time, the tertiary structure of the peptide has not been fully elucidated. NMR and CD studies of the intact peptide and fragments of it suggest that the *C*-terminal part (residues 15–28) has an α-helical conformation and that the *N*-terminal part is not stabilized by secondary structure.[3,4]

Peptides belonging to the glucagon–secretin family have, to various extents, been shown to compete with VIP for binding to the VIP-receptor site. PHI (peptide with *N*-terminal histidine and *C*-terminal isoleucine amide) and secretin compete with VIP for binding with IC_{50} values of about 20 nM and about 200 nM, respectively, while glucagon does not displace VIP at all in rat liver plasma membranes. The IC_{50} value for VIP lies between 0.2 and 3.2 nM[5–7] in the same tissue.

The aim of the present study was to search, in fractions of pig intestinal peptides, for additional peptides able to bind to the VIP receptor. We here describe the isolation of a peptide which competes with VIP for binding to the receptor site on liver plasma membranes with an IC_{50} value of 2.8 μM. The peptide was identified as the *N*-terminal part (residues 1–28) of triosephosphate isomerase (TIM). Since the amino acid sequence of the enzyme fragment is almost totally unrelated to that of VIP, we propose that the three-dimensional structure of VIP may resemble that of TIM(1–28), at least when it is in contact with the plasma membrane for binding to the receptor.

Results

Structural analysis. The amino acid composition of the purified peptide was determined and is shown in Table 1. The peptide was cleaved with CNBr to yield two major frag-

Table 1. Amino acid compositions of TIM(1–28) and its CNBr fragments. Values are molar ratios obtained after total hydrolysis of the peptides. Values within parentheses are those from the sequence analysis. The value for Ser has been corrected by subtracting the background. ND = not determined.

Amino acid	TIM(1–28)	*N*-terminal CNBr fragm.	*C*-terminal CNBr fragm.
Asx	4.2 (5)	1.3 (1)	2.3 (4)
Thr	0.9 (1)	0.2 (–)	0.8 (1)
Ser	0.3 (–)	0.7 (–)	– (–)
HoSer	– (–)	0.4 (1)	– (–)
Glx	1.4 (1)	0.9 (–)	1.1 (1)
Pro	1.0 (1)	1.3 (1)	– (–)
Gly	4.0 (4)	2.3 (2)	2.2 (2)
Ala	2.0 (2)	1.6 (2)	– (–)
Val	1.0 (1)	0.7 (1)	– (–)
Met	1.4 (1)	– (–)	
Ile	1.4 (1)	– (–)	0.8 (1)
Leu	2.8 (3)	– (–)	2.5 (3)
Phe	1.8 (2)	1.6 (2)	– (–)
Trp	ND (1)	ND (1)	ND (–)
Lys	2.6 (3)	1.1 02)	1.3 (1)
Arg	1.7 (2)	0.5 (1)	1.3 (1)
Total	28	14	14

TIM(1–28)

```
1                5                  10                  15                  20                  25
Ala-Pro-Ala-Arg-Lys-Phe-Phe-Val-Gly-Gly-Asn-Trp-Lys-Met-Asn-Gly-Arg-Lys-Asn-Asn-Leu-Gly-Glu-Leu-Ile-Asn-Thr-Leu
 D  D  D  D  D  D  D  D  D  D  D                                    D  D  D  D  D  D  D
```

VIP

```
1                5                  10                  15                  20                  25
His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn*
```

Fig. 1. The amino acid sequence of the porcine *N*-terminal TIM fragment as established after sequencer degradation of 2 nmoles of the intact peptide. The repetitive yield calculated on Gly(9–22) was 96 %. Residues also identified by manual sequence analysis with the DABITC method of the intact peptide (residues 1–5) and tryptic fragments (residues 6–11 and 18–24) are indicated by the letter D.

ments. The amino acid compositions of these fragments are also shown in Table 1. The amino acid sequence of the whole peptide and the *C*-terminal CNBr fragment were both determined and the sequence is shown in Fig. 1. There are no ambiguities in the sequence determinations and thus, despite some minor discrepancies between the sequence analysis and the amino acid composition data, the sequence in Fig. 1 appears to be correct. A search for homologies to amino acid sequences in the NBRF data base showed a high similarity with the *N*-terminal (1–28) sequences of several triosephosphate isomerases (EC.5.3.1.1.). Obviously, the isolated peptide corresponds to the thus far unknown sequence of the porcine form of this enzyme fragment. There are four amino acid substitutions in the *N*-terminal part of the porcine enzyme compared with the human form:[8] at position 3 (Ser→Ala), 19 (Gln→Asn), 20 (Ser→Asn) and 26 (Gly→Asn). The three former substitutions were also confirmed by manual sequencing of tryptic peptides using the DABITC method (Fig. 1). Furthermore, porcine TIM, amino acid residues 1–28, was synthesized by the solid-phase method and shown to coelute with the purified peptide on reversed-phase HPLC.

The secondary structural properties, according to Chou and Fasman,[9] showed no close relationships between VIP and TIM(1–28). However, the hydrophilicity pattern of the two peptides, determined according to Kyte and Doolittle,[10] show a similar pattern (Fig. 3), especially if the sequences are shifted 3 positions relative to each other.

Binding study. The synthesized TIM(1–28)-fragment was tested for binding in the VIP-RRA. As shown in Fig. 2, the enzyme fragment competes with VIP for binding to the VIP receptor site. Half-maximal binding is obtained at 2.8 μM, a concentration about 1000 times higher than for VIP. The isolated, natural fragment was also tested in the VIP-RRA at concentrations up to 10^{-5} M and gave similar results (IC_{50} = 2.2 μM, data not shown). At these concentrations, peptides not related to VIP, such as CCK (tested at 10^{-5} M) and insulin (10^{-4} M), do not displace VIP. On the other hand, PHI and secretin displace VIP at about 10 and 100 times higher concentration, respectively, than VIP (Fig. 2).

Discussion

The finding that a peptide which displaces VIP at the receptor site almost completely at 10^{-4} M concentration turned out to be the *N*-terminal part of TIM, the amino acid sequence of which is not related to that of VIP, was surprising. However, it has been shown, in the case where parathyroid hormone competes with glucagon for binding to the glucagon receptor site in the liver, that unrelated peptides can compete for the same receptor site.[11] The common conformational features of the ligands may be more important for receptor recognition than the amino acid sequence homology. This may be particularly interesting as regards structure similarities between peptides binding to a membrane-bound receptor, since the structure of the peptide when it is in contact with the membrane may be modified and stabilized compared with the soluble state.

The three-dimensional structure of TIM has been determined[12] and is well established. It can be compared with what is known about the conformation of VIP. TIM is built up of a β/α-barrel structure forming a cylinder of eight inner β-sheet strands connected by α-helical strands running antiparallel to the β-strands on the outside of the cylinder.[12] The first 31 residues of TIM correspond to one (βα)-'unit' of the enzyme. Studies on the chicken TIM show that the β-structure is found in residues 6–12 and that the α-helical structure is present from residue 17–31. The isolated porcine TIM fragment with its 28 amino acid residues thus almost corresponds to one (βα)-'unit' of the enzyme.

VIP has not been crystallized. Theoretical calculations using the rules of Chou and Fasman for the prediction of protein secondary structure[9] indicate that the *C*-terminal half of VIP has a propensity to adopt an α-helical structure. In water VIP retains considerable conformational freedom as shown by CD and ORD spectral analysis.[3,13,14] CD and NMR studies in different organic solvents or mixtures of organic solvents and water, suggest that VIP has a propensity to adopt a considerable degree of α-helical conformation in these solvents[3,13–15] and also that a *C*-terminal fragment of VIP (VIP 15–28) could form an α-helical structure.[3] The ability of VIP to adopt secondary structure in

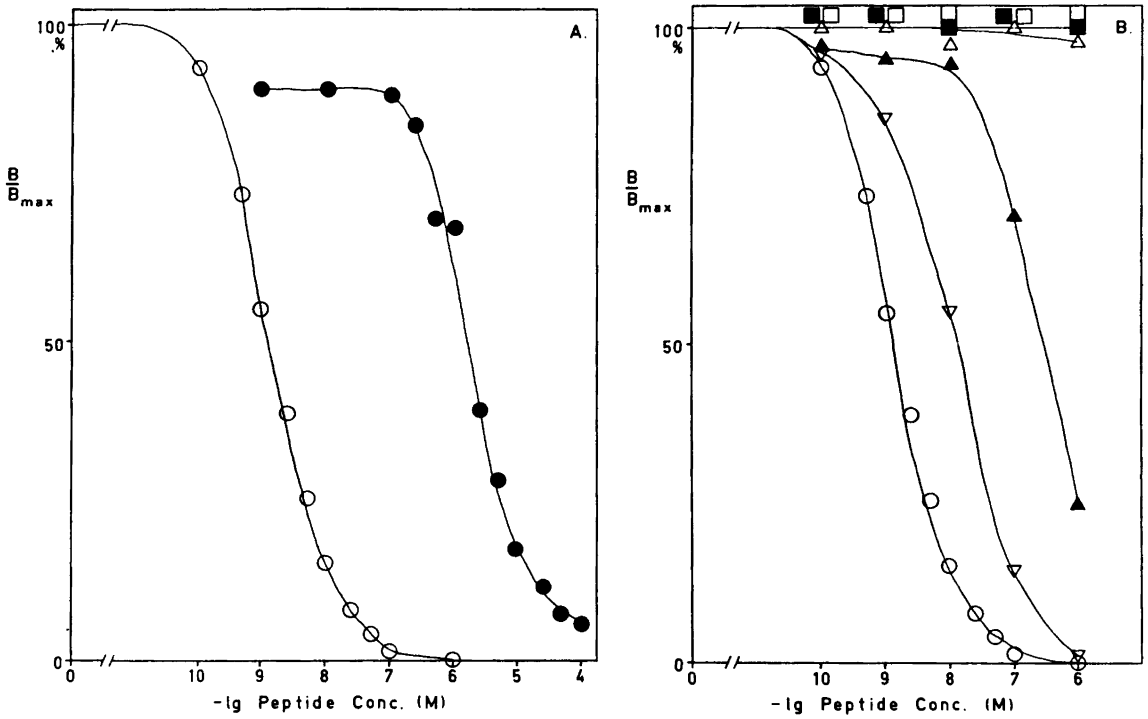

Fig. 2. A, Inhibition of ^{125}I-VIP binding to rat liver plasma membranes by increasing concentrations of VIP (○) and synthetic TIM fragment(1–28) (●); B, Inhibition of ^{125}I-VIP binding to the same membrane preparation as in A by increasing concentrations of VIP (○), PHI (▽), secretin (▲), glucagon (△), CCK (■) and insulin (□).

Results are expressed as percentages of ^{125}I-VIP bound in the presence versus absence of added peptide. Specific binding of ^{125}I-VIP was defined as the portion that could be displaced in the presence of 1 μM VIP. Values at each concentration point were determined in triplicate.

organic solvents may be of some relevance when the peptide is in contact with the hydrophobic environment at the cell membrane.

The conformation of the TIM(1–28) fragment may be relatively stable since it forms a defined structural unit, and it shares some properties with the structure of VIP. Thus, the *C*-terminal part of both the enzyme fragment and VIP has (or may adopt) an α-helical structure. The hydrophilicity curves of VIP and TIM(1–28), according to Kyte and Doolittle,[10] show similarities (Fig. 3). This may be of certain importance considering interactions at the cell membrane level. Another interesting feature is that TIM(1–28) has a Phe-residue at position 6 and this property is shared with all the members of the glucagon–secretin family of peptides suggesting some, as yet unrevealed, importance of this residue. The number of amino acid residues in the TIM fragment and VIP is also identical.

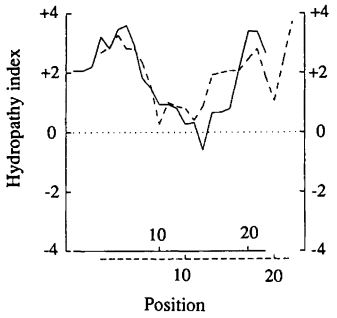

Fig. 3. Hydropathy plot according to Kyte and Doolittle of TIM (1–28) (———) and VIP (- - - - -) using six residue spans. Each position corresponds to the first amino acid residue in a six-residue span. The curve of VIP is shifted three positions to the right compared with the curve of TIM(1–28).

The sequence of TIM(1–28) contains a typical prohormone processing signal sequence: a pair of basic residues (Arg-Lys) at positions 4–5 and an amidation signal (Gly-Arg-Lys) at positions 16–18. Whether this is of any importance is doubtful since TIM is an intracellular enzyme and the TIM mRNA[8] does not code for the signal sequence required if the protein synthesized is to be brought into contact with the processing enzymes situated in the secretory granules.

In conclusion, the binding of the *N*-terminal fragment of TIM to the VIP receptor is an example which strongly suggests that the overall conformation of the ligand, rather than just the amino acid sequence, must be taken in account when discussing receptor interaction, antagonist design, etc. In the case of VIP, a hydrophilicity pattern similar to that in TIM(1–28) is observed. Furthermore, the two peptides share *C*-terminal structural features while both peptides have a propensity to form α-helical structures in the *C*-terminal part.

Experimental

Materials. ^{125}I-VIP (3-Iodotyrosyl-^{125}I-VIP) was from Amersham, UK. Exclusion chromatography was carried out on Sephadex G-25 (Pharmacia, Uppsala, Sweden) and ion exchange chromatography on CM-cellulose (CM-22, Whatman, Kent, UK). Preparative high performance liquid chromatography (HPLC) was performed on a preparative HPLC system from Waters Assoc. using a Vydac C18 column (22×250 mm, 15–20 μm particle size) and analytical HPLC was performed on a Waters instrument with an LKB Ultropac TSK ODS-120T column (7.8×300 mm, 10 μm particle size) or an LKB Ultropac TSK 535 CM column (7.8×150 mm). VIP, secretin, PHI and cholecystokinin (CCK)-33 were natural peptides purified from porcine upper intestine. Insulin and glucagon were from Novo (Denmark).

Purification. The extraction procedure has been described in detail elsewhere.[16] Briefly, 500 g of a concentrate of thermostable intestinal polypeptides were dissolved in 4 l water containing 0.5 % thiodiglycol after which 18 l of isopropyl alcohol were added. A precipitate (F-0) was formed and removed by centrifugation. To the filtrate were added 18 l of cold isopropyl alcohol and after 24 h a precipitate (F-1) which formed at −20 °C was removed by filtration. The filtrate was adjusted to pH 6.0 with 0.5 M potassium acetate and left for another 24 h at −20 °C. The precipitate formed was collected by filtration, dried and suspended in water. The suspension was adjusted to pH 3 with 3 M acetic acid and saturated with NaCl. The salt precipitate, called F-2, typically weighed about 60 g. Throughout the following purification, fractions were tested for VIP-receptor binding in the VIP-RRA.

Fraction F-2 (247 g, corresponding to four isopropyl alcohol fractionations) was applied to a Sephadex G-25 column (35×135 cm) and chromatographed in 0.2 M acetic acid. Five fractions were collected and precipitated with NaCl. Fraction 4 (12 g, eluted with 28–40 l 0.2 M acetic acid) was applied to a second Sephadex G-25 column (10×100 cm) and the chromatography was carried out in 0.2 M acetic acid. Four fractions were collected and lyophilized.

Sephadex fraction 4 (600 mg, eluting just before the salt, with 2.1–3 l 0.2 M acetic acid) from the second Sephadex chromatography was subjected to CMC chromatography. The column (2.5×30 cm) was equilibrated with 0.01 M NH_4HCO_3 and elution was carried out with 0.01, 0.02, 0.1 and 0.2 M NH_4HCO_3 and 0.2 M NH_3. All fractions collected were lyophilized.

Fraction 9 (33.7 mg), eluted with 0.2 M NH_4HCO_3 by CMC chromatography, was fractionated on a preparative reversed-phase HPLC column (Vydac 22×250 mm). Elution was carried out with a gradient of 25→45 % acetonitrile in water with 0.1 % TFA over 30 min at a flow rate of 15 ml min^{-1}. Fraction 9, which eluted after 12–14 min, was subjected to a second reversed-phase HPLC fractionation. To an Ultropac TSK ODS-120T column (7.8×300 mm) were applied 5×600 μg and then fractions were eluted under isocratic conditions: 29 % acetonitrile in water with 0.1 % TFA, at a flow rate of 1.5 ml min^{-1}. Fraction 1, eluting after 14–17 min was finally subjected to a CM–HPLC chromatography using an Ultropac TSK 535 CM column (7.5×150 mm) with an elution system of 0.2 M acetic acid as buffer A and 1 M ammonium acetate in 0.2 M acetic acid as buffer B[17] and a gradient of 30→60 % buffer B over 30 min at a flow rate of 1 ml min^{-1}. Fraction 1 from this chromatography was found to be essentially pure as judged by rechromatography on reversed-phase HPLC and by the structural analysis which confirmed one amino acid sequence.

Structural analysis. Amino acid compositions were determined with a Beckman 121 M analyser after hydrolysis of the peptide in evacuated tubes with 6 M HCl containing 0.5 % phenol at 110 °C for 24 h. CNBr cleavage was carried out in 70 % formic acid for 24 h at room temperature. For 3 nmol peptide, 40 mg CNBr (Merck) was used. Amino acid sequences were determined by degradation of the peptide in an Applied Biosystems 470A gas-phase sequencer and analysis of phenylthiohydantoin derivatives by HPLC on a Nucleosil C18-column with an acetonitrile gradient in sodium acetate.[18,19] The manual dimethylaminoazobenzene isothiocyanate (DABITC) sequencing method[20] was also used.

Radio-receptor assay (RRA). Liver plasma membranes were prepared from male Wistar rats (150–250 g) according to the method of Neville.[21] Protein concentration was determined by means of the Bradford method.[22] For binding studies, 60 μg liver plasma membrane proteins were incubated together with ^{125}I-VIP (ca. 25 pM final concentration) and samples at various concentrations in a final volume of 250 μl. The assay buffer was a 100 mM Tris, pH 7.5 with 3 % BSA and 1 mg ml^{-1} bacitracin. The incubation was carried out at 30 °C for 10 min after which VIP bound to the membranes was separated from unbound VIP by centrifugation at 10000 g for 5 min. Finally, the radioactivity retained in the pellets was determined with a Beckman gamma-counter with a counting efficiency of 64 %. Porcine VIP competitively inhibited the binding of ^{125}I-VIP in the range 10^{-10}–10^{-6} M and half-maximal inhibition was observed at 1.2 nM VIP.

Peptide synthesis. Peptide synthesis was carried out in an automatic mode using an Applied Biosystems Model 430A Peptide Synthesizer. To a ready-made PAM-resin with coupled t-Boc-Leu (Applied Biosystems) were added, successively, activated (symmetric anhydrides or hydroxybenzotriazole esters for Asn and Arg) side-chain protected t-Boc amino acids according to a standard program.

The completed 28-residue peptide was cleaved from the resin and deprotected by acidolysis with HF–anisole–dimethyl sulfide–*p*-thiocresol (10:1:1:0.2) at 0 °C for 60 min,

followed by extraction with 30 % acetic acid/ether. To ensure complete deprotection of the Trp residue, the lyophilized crude preparation was deformylated in 6 M guanidine hydrochloride containing 1 M ethanolamine at 0 °C for 5 min. UV spectral analysis of the deformylated product showed a strong absorbance at 280 nm relative to 300 nm, which confirmed that the reaction had gone to completion.

The deformylated peptide was purified by HPLC; first on a preparative reversed-phase HPLC column (Vydac 22×250 mm) eluted with 20→40 % acetonitrile in water with 0.1 % TFA over 60 min at a flow rate of 10 ml min^{-1} and then on an LKB Ultropac ODS 120T column (7.8×300 mm) eluted with 28→38 % acetonitrile in water with 0.1 % TFA over 25 min at a flow rate of 1.5 ml min^{-1}. The purified synthetic peptide was found to coelute with the natural TIM(1–28) fragment in the HPLC system and the amino acid composition was consistent with that expected for a correct replica (Asx, 4.9; Thr, 1.0; Glx, 1.1; Pro, 0.8; Gly, 4.0; Ala, 1.7; Val, 1.1; Met, 1.2; Ile, 1.0; Leu, 3.3; Phe, 1.7; Trp, 0.5; Lys, 3.1; Arg, 2.0) of the natural peptide. The overall yield of the purified synthetic TIM(1–28) fragment was approximately 10 %.

Hydropathy plots. Hydropathy plots were constructed according to Kyte and Doolittle[10] using 6-residue spans.

Acknowledgements. This work was supported by grants from the Swedish Medical Research Council (project 13X-1010 and 03X-3532), *Karolinska Institutet,* Bengt Lundqvists Foundation, Lars Hiertas Foundation, KabiGen AB and Skandigen AB.

References

1. Said, S. I. and Mutt, V. *Eur. J. Biochem. 28* (1972) 199.
2. Said, S. I. and Mutt, V. In: Said, S. I. and Mutt, V., Eds., *Vasoactive Intestinal Peptide and Related Peptides, Annals NY Acad. Sci.*, Vol. 527, New York 1988.
3. Fournier, A., Saunders, J. K. and St-Pierre, S. *Peptides 5* (1984) 169.
4. Fournier, A., Saunders, J. K., Boulanger, Y. and St-Pierre, S. A. In: Said, S. I. and Mutt, V., Eds., *Vasoactive Intestinal Peptide and Related Peptides, Annals NY Acad. Sci.,* Vol. 527, New York 1988, pp. 51–67.
5. Desbuquois, B. *Eur. J. Biochem. 46* (1974) 439.
6. Fahrenkrug, J., Gammeltoft, S., Staun-Olsen, P., Ottesen, B. and Sjöquist, A. *Peptides 4* (1983) 133.
7. Robberecht, P., Coy, D. H., De Neef, P., Camus, J.-C., Cauvin, A., Waeldebroeck, M. and Christophe, J. *Eur. J. Biochem. 159* (1986) 45.
8. Maquat, L. E., Chilcotes, R. and Ryan, P. M. *J. Biol. Chem. 260* (1985) 3748.
9. Chou, P. Y. and Fasman, G. D. *Adv. Enzymol. 47* (1978) 45.
10. Kyte, J. and Doolittle, R. F. *J. Mol. Biol. 157* (1982) 105.
11. Shah, G. V., Epand, R. M. and Orlowski, R. C. *Mol. Cell. Endocrinol. 49* (1987) 203.
12. Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Podgson, C. I., Wilson, I. A., Corran, P. H., Furth, A. J., Milman, J. D., Offord, R. E., Priddle, J. D. and Waley, S. G. *Nature (London) 255* (1975) 609.
13. Bodanzky, M., Bodanzky, A., Klauser, Y. S. and Said, S. I. *Bioorg. Chem. 3* (1974) 133.
14. Robinson, R. M., Blakeney, E. W., and Mattice, W. L. *Biopolymers 21* (1982) 1217.
15. Fry, D. C., Madison, V. S., Bolin, D. R., Toome, V. and Wegrzynski, B. B. *Biochemistry 28* (1989) 2399.
16. Chen, Z.-W., Agerberth, B., Gell, K., Andersson, M., Mutt, V., Östenson, C.-G., Efendic, S., Barros-Söderling, J., Persson, B. and Jörnvall, H. *Eur. J. Biochem. 174* (1988) 239.
17. van den Eijden-van Raaij, A. J. M., Koorneef, I., van Oostwaard, Th. M. J., de Laat, S. W. and van Zoelen, E. J. J. *Anal. Biochem. 163* (1987) 263.
18. Zimmermann, C. L., Appella, E. and Pisano, J. J. *Anal. Biochem. 77* (1977) 569.
19. Kaiser, R., Holmquist, B., Hempel, J., Vallee, B. L. and Jörnvall, H. *Biochemistry 27* (1988) 1132.
20. Chang, J. Y., Brauer, D. and Wittmann-Liebold, B. *FEBS Lett 93* (1978) 205.
21. Neville, D. M., Jr. *Biochim. Biophys. Acta 154* (1968) 540.
22. Bradford, M. *Anal. Biochem. 72* (1976) 248.

Received May 16, 1990.