# INACTIVATION OF SOMATOSTATIN (GH-RIH) AND ITS ANALOGS BY CRUDE AND PARTIALLY PURIFIED RAT BRAIN EXTRACTS

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## 1. Introduction

Somatostatin, a cyclic tetradecapeptide, inhibits the release of growth hormone from the pituitary and glucagon and insulin from the pancreas of fasted mammals [1,2]. Its value as a therapeutic agent is severely limited, however, by its extremely short biological half life of approx. 4 min [3, 4]. As in the case of other peptidyl hormones there is considerable interest in the preparation of longer acting derivatives which could have application to the treatment of diabetes and developmental disorders. For the preparation of synthetic analogs a knowledge of the mechanisms of inactivation in brain and tissues could facilitate a more rational approach as illustrated recently for other hypothalamic releasing factors. In the case of LH-RH (luteinising hormone releasing hormone) it was shown that a simple substitution of an internal residue by D-Ala blocked the action of an endopeptidase with a resultant increase in the biological activity in vivo [5]. This particular study introduced a new concept, namely, that structure as related to biodegradation is equally valid to the traditional approach involving considerations of structure-receptor relationships. Analogs with improved stability in presence of serum or tissue enzymes are more likely to reach the target sites involved in hormonal action and this may account for their longer action.

Somatostatin unlike LH-RH is characterised by a free N-terminal grouping and by the presence of a disulfide bridge spanning residues 3–14 [1]. As such, it is a potential substrate for the action of aminopeptidases present in crude extracts but the role of the ring structure in breakdown is unexplored. It has been established in the case of other cyclic hormones, notably oxytocin and vasopressin, that the ring acts as a steric hindrance to aminopeptidases and some endopeptidases; these hormones are inactivated in brain, however, by novel C-terminal cleaving enzymes acting on the tripeptidyl alicyclic tails [6,7]. With the objective of delineating the enzymes involved in breakdown of somatostatin, this hormone and its cyclic and linear analogs were incubated with crude and partially purified enzyme from rat brain since these extracts are known to contain a spectrum of enzymes cleaving a variety of peptidyl hormones [8].

## 2. Materials and methods

Somatostatin (GH-RIH, growth hormone releasing hormone) and its cyclic and linear analogs were supplied as a gift by Jean Rivier of the Salk Institute, San Diego, California. These included the native form (fig.1) the reduced form $H_2$ somatostatin ($H_2$ GH-RIH), and two analogs des(Ala$^1$–Gly$^2$)-N-acetyl-Cys$^3$ GH-RIH and des(Ala$^1$-Gly$^2$)-*N*-acetyl-Cys$^3$ $H_2$ GH-RIH. At the concentrations used all were completely soluble in water or buffer at pH 7.6

Brain extracts were prepared from the rat by homogenization in 10 vols of cold 0.32 M sucrose containing 0.1 mM Cleland's reagent and then centrifuged at 100 000 *g* for 1 hr to prepare the supernatant fractions. Enzyme was purified batchwise using 10 ml of supernatant which was applied to a column of DEAE-cellulose 25 × 2 cm equilibrated with a 40 mM Tris–HCl buffer pH 7.6 containing 0.1 mM Cleland's reagent. Enzyme was eluted stepwise with a salt gradient 0.1–0.3 M NaCl. All fractions were tested for proteinase activity with hemoglobin using the method



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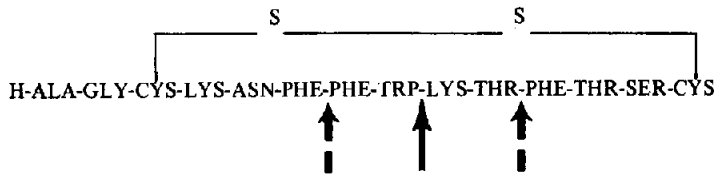


Fig.1. Amino acid sequence of somatostatin (GH-RIH). The arrows indicate probable sites of action of brain neutral proteinase based on the release of residues by rat brain extracts after 1 hr incubation (see table 1).

of Marks et al. [9] and the active fractions then tested with hormone. Incubations with hormone generally consisted of 0.1 ml of homogenate (equivalent to 1 mg protein) or 0.1 ml supernatant (0.3 mg protein) or 0.5 ml of the purified enzyme fraction (0.1 mg) eluted with 0.1–0.2 M NaCl from the DEAE-cellulose column, to which was added 50 nmol of hormone in a vol of 0.2 ml of water, and 0.1 ml of 40 mM Tris–HCl buffer. The reaction was terminated by addition of an equal volume of 6% w/v sulfosalicylic acid and then centrifuged at 2 000 *g*, and the supernatant retained. Breakdown was evaluated from the release of free amino acids as detected by a modified method on the Technicon autoanalyzer as previously described [8]. It was shown in earlier studies that a large numbre of peptidyl intermediates have low color values relative to norleucine and do not interfere [5–8]. All reactions were carried out in triplicate with suitable controls.

### 3. Results and discussion

Incubation of the native form of somatostatin with rat brain homogenate or its supernatant fraction led to the release within 3 hr at 37°C of all the constituent amino acids (table 1). The release of the internal amino acids (Phe, Lys, Trp, Thr) in larger amounts as compared to the N- and C-terminal (Ala, Gly, Ser and Cys) point to an internal cleavage as a rate-limiting step. Crude extracts contain a spectrum of enzymes so that there is a secondary release of amino acids resulting from the action of exopeptidases acting on the split fragments. The preferential release of internal amino acids by a 'neutral endopeptidase' is thus similar to that reported for bradykinin [10], LH-RH [5] and substance P [11]. An indication of the probable sites for cleavage was provided by a time study (table 1). With native somatostatin at 1 hr the only amino acids detected were Phe and Lys implying that bonds adjacent to these residues were involved (fig.1). At 3 hr, all amino acids residues were detected in varying proportions (table 1) with a maximum breakdown of about 40% for the internal residues. Based on the appearance of Phe this rate of breakdown is equivalent to 1.4 μmol per g fresh weight per hr. Incubation for longer periods did not significantly increase the release of amino acids. This could indicate that a number of the intermediate products are poor substrates for brain exopeptidases or that the enzyme involved is labile. Neutral proteinases of brain are known to be labile and the present observations are in accordance with previous studies using LH-RH and substance P [5, 11].

Incubation of the linear (reduced) hormone ($H_2$-GH-RIH) with brain supernatant gave a similar pattern of release except for the earlier appearance of Thr at 1 hr as compared to the cyclic hormone (table 1). This could indicate an additional cleavage site for the brain endopeptidase. These data show that the ring structure does not play a significant role in rates of breakdown. This result is in striking contrast to oxytocin, a cyclic nonapeptide which is unaffected by crude and purified brain aminopeptidases and endopeptidases: removal of the disulfide bridge, however, leads to a very rapid degradation [6, 7]. Although somatostatin has a free N-terminal alanine its low rate of release at the shorter incubation periods indicates that this is not a major route of inactivation. It is known, however, that the N-terminal dipeptide in any case is not essential for biological activety [4]. It might be concluded that linear analogs lacking Ala–Gly have a greater likelihood for better activity in addition to involving simpler synthetic procedures.

Studies on two of the available analogs support the earlier conclusion for the presence in brain of a

Table 1
Cleavage of somatostatin (GH-RIH) by crude and partially purified enzyme from rat brain

| Substrate | Time (hr) | nmoles per cent released | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | H-Ala[1][a] | Gly[2] | Cys[3,14] | Lys[4,9] | Asn[5] | Phe[6,7,9] | Trp[8] | Thr[10,12] | Ser[13] |
| GH-RIH | 1 | 0 | 0 | 0 | 11 | 0 | 23 | 0 | 0 | 0 |
| | 3 | 29 | 17 | 13 | 37 | 37 | 37 | 37 | 36 | 24 |
| | 20 | 47 | 31 | 20 | 33 | 44 | 40 | 40 | 40 | 33 |
| | 20[b] | 0 | 0 | tr | 28 | tr | 32 | 6 | 21 | 16 |
| $H_2$ GH-RIH | 1 | 0 | 0 | 0 | 16 | nd | 19 | 0 | 10 | tr |
| | 3 | 24 | 8 | tr | 27 | 48 | 35 | 16 | 31 | 40 |
| | 20 | 31 | 43 | 15 | 55 | 51 | 47 | 30 | 50 | 29 |
| des ($Gly^1-Ala^2$) N-acetyl-$Cys^3$ GH-RIH | 3 | – | – | 0 | 33 | 37 | 36 | nd | 41 | 5 |
| des($Gly^1-Ala^2$) *N*-acetyl-$Cys^3$ $H_2$-GH-RIH | 3 | – | – | 0 | 36 | 43 | 55 | 43 | 52 | 10 |

[a] Numbers refer to the position of residues as indicated in fig.1.

[b] Purified enzyme eluted from a DEAE-cellulose column by 0.1–0.2 M NaCl as described in the Methods section. nd=not determined. tr = trace quantities. The incubation mixture consisted of 0.1 ml of a 100 000 *g* supernatant of rat brain prepared in 10 vols of 0.32 M sucrose containing 0.1 ml Cleland's reagent, 50 nmol of hormone in a vol of 0.2 ml water, and 0.1 ml of 40 mM Tris–HCl pH 7.6. For purified enzyme 0.5 ml of the active fraction (0.1 mg protein) was used. The reaction was terminated with an equal vol of 6% w/v sulfosalicylic acid and breakdown products determined by amino acid analysis. Values represent the mean of 2–4 determinations.

Table 2
Partial purification of the enzyme inactivating GH-RIH

| Fraction | Protein (mg) | Activity (nmoles Phe) | Specific activity (nmoles Phe/mg pr.) | Purification |
|---|---|---|---|---|
| Homogenate | 110 | 4350 | 39 | 1 |
| Supernatant 100 000 *g* | 23 | 3700 | 161 | 4 |
| DEAE-cellulose 0.1–0.2 M NaCl | 5 | 1400 | 280 | 7.2 |

Enzyme was purified batchwise using 10 ml of supernatant prepared in 0.32 M sucrose containing 0.1 mM Cleland's reagent as described in the Methods Section. Enzyme was applied to a column of DEAE-cellulose 25 × 2 cm equilibrated with the same buffer and eluted stepwise with 0.1, 0.2 and 0.3 M of NaCl. Breakdown was evaluated from the release of Phe in a 3 hr incubation.

rate-limiting endopeptidase. Incubation of the analog lacking the N-terminal dipeptide and with a blocked Cys at position 3, des(Ala–Gly)-*N*-acetyl $Cys^3$ GH-RIH, with brain supernatant again led to a preferential release of internal amino acids. In this case the role of an aminopeptidase in the initial cleavage can be completely excluded. Also the poor release of Ser would further exclude a major role for carboxypeptidases acting on the intact hormone. As in the case of the intact hormones the absence of a disulfide ring in the analog des(Ala–Gly)-*N*-$Cys^3$ $H_2$ GH-RIH did not significantly alter the release of internal amino acids relative to those of the N- and C-terminal. These two analogs are reported to have a more sustained action in vivo [4] but this may be more related to their insolubility requiring administrations as a suspension leading to a sustained release.

Brain 'neutral endopeptidases' are exremely labile and as a consequence have never been adequately characterized [12]. Partial purification of the brain enzyme inactivating somatostatin was achieved by passage through a short column of DEAE-cellulose as described briefly in the Methods section. The supernatant was 4-fold and the enzyme eluted with 0.1–0.2 M NaCl was approx. 7-fold enriched as compared to that of the homogenate. The active fractions were characterised by high rates of breakdown when incubated with protein substrates such as hemoglobin and histone. This finding thus supports the contention that a neutral proteinase is involved but additional studies are required to determine if it is identical to that inactivating LH-RH, bradykinin and substance P [5,10,11].

## 4. Conclusions

Somatostatin is cleaved by brain extracts with the release in varying amounts of all the constituent amino acids. The relative rates for release of Phe, Lys, Trp and Thr (internal amino acids) to that for Ala, Gly, Ser, and Cys (N- and C-terminal) point to the acion of a neutral endopeptidase as playing a rate-limiting role. Studies with analogs indicated that the presence or absence of a disulfide ring did not significantly alter rates of breakdown. The slower removal of Ala at short incubation periods showed that somatostatin is not a good substrate for brain aminopeptidases. The lower release of Cys and Ser similarly indicated that carboxypeptidases do not represent a major route for breakdown. Studies with analogs with a blocked N-terminal cysteine confirm the mechanism for the action of an endopeptidase. The enzyme involved is labile and has the characteristics associated with a brain neutral proteinase. These studies indicate the need to prepare new derivatives in the linear-form having D-amino acids or other substituents at the positions of the internal amino acids in order to block the rate-limiting sites of inactivation.

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