Evidence for presence of peptide α -amidating activity in pancreatic islets from newborn rats

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The peptide α -amidating activity of a homogenate of pancreatic islets from 5–7-day-old rats was investigated, using as substrate a glycine-extended tripeptide (D-Tyr-Val-Gly). The islet homogenates had a marked amidating activity, with a K_m of 57 μ M, a V_{max} of 185 pmol/h per mg and a pH optimum of 7.0. This activity was dependent on the presence of ascorbic acid (in the reduced form) and Cu^{2+} , the optimum concentrations being 4 mM and 40 μ M respectively. On fractionation of the homogenate, the highest specific activity was found in the soluble fraction. Exocrine pancreatic tissue showed very low levels of amidating activity.

INTRODUCTION

About half of all biologically active peptides are amidated at their C-termini by a peptidylglycine α -amidating mono-oxygenase (PAM; EC 1.14.17.3). High activities of this enzyme have been demonstrated in different tissues, e.g. in rat heart atrium (Stoffers & Eipper, 1990) and in intermediate lobes of bovine hypophysis, where it is present in A and B forms (Murthy *et al.*, 1986). The enzyme has been cloned (Eipper *et al.*, 1987; Ohsuye *et al.*, 1988).

As it catalyses the last in a series of processing steps, PAM may have an essential role in regulating the final processing of active peptides (Eipper *et al.*, 1987). Its activity is influenced by various endocrine and pharmacological factors, such as glucocorticoids and thyroid hormone (Mains & Eipper, 1984; Mains *et al.*, 1985) and is very different in different tissues (Stoffers & Eipper, 1990). One way of regulating the activity of PAM, e.g. by hormones, might be through a regulation of the availability of its cofactors, which are ascorbic acid and Cu^{2+} (Thorn *et al.*, 1988). In an attempt to compare the characteristics of PAM regulation in different tissues, we have previously studied uptake of ascorbic acid into pancreatic islet cells. This transport in many ways resembles that found in the neurohypophysis and pars intermedia of the hypophysis. It differs, however, in being stimulated slightly by glucose (Zhou *et al.*, 1989).

Several amidated peptides are present in pancreatic islets, such as thyrotropin-releasing hormone (TRH), pancreastatin and pancreatic polypeptide. A few studies have been published concerning the possible relationship between the amidation reaction of peptide hormones and pancreatic function (Paquette *et al.*, 1981; Ouafik *et al.*, 1987). Ouafik *et al.* (1986, 1987) have demonstrated previously the presence of high PAM activity in whole pancreatic tissue from neonatal rats. Treatment of the rats with streptozotocin decreased the PAM activity in a crude mitochondria/secretory granule fraction by 50%. The activity studied by Ouafik *et al.* (1986, 1987) paralleled the concentration of TRH during the first days after birth, but apart from that it was not characterized. In the present paper we report direct studies of PAM activity and some of its characteristics in pancreatic islets from newborn rats.

MATERIAL AND METHODS

Preparation of pancreatic islets and exocrine pancreatic tissue

Pancreatic tissue from 5–7-day-old Wistar rats was rinsed with ice-cold Hanks' buffer, pH 7.4, and the tissue was then treated with 5 mg of collagenase/ml (Boehringer; 0.244 units/mg). Pancreatic islets were separated manually from exocrine tissue (Brunstedt *et al.*, 1984). After separation, the islets were washed twice with ice-cold 100 mM-Tes (pH 7.0), subjected to three cycles of freezing–thawing and then homogenized using a Teflon/glass homogenizer. The homogenate was suspended in 100 mM-Tes, pH 7.0, and used for amidation assays. In some experiments, soluble cytosolic and membrane fractions were prepared by centrifugation of the islets (90 000 *g* for 10 min) after freezing and thawing (three cycles). Exocrine tissue was collected during the separation of islets and treated in the same way as islet tissue for amidation assays.

Assays for amidation activity

The assay was performed at 37 °C in the presence of a constant atmosphere (O_2/CO_2 , 19:1), using the method of Eipper *et al.* (1983a). D-Tyr-Val-Gly (Bachem) or ^{125}I -labelled D-Tyr-Val-Gly was used as substrate and D-Tyr-Val-NH $_2$ or ^{125}I -D-Tyr-Val-NH $_2$ respectively were the products measured. The reaction mixture (pH 7.0; 40 μ l in most cases) contained 100 mM-Tes, 100 μ g of catalase/ml (Boehringer), approx. 100 μ g of tissue protein, and various amounts of ascorbic acid, $CuSO_4$ and D-Tyr-Val-Gly or ^{125}I -D-Tyr-Val-Gly as indicated in the Results section. The reaction was stopped by adding 1.5 ml of ice-cold 10 mM-sodium phosphate (pH 5.0) to the reaction mixture or by quickly freezing it. For the experiments on pH dependence, 100 mM-Tris/HCl/base buffer was employed.

The purity of purchased tripeptide and bipeptide amide were checked by h.p.l.c. Both peptides were of high homogeneity, so they were used directly in amidation assays. Iodination of D-Tyr-Val-Gly and D-Tyr-Val-NH $_2$ was carried out using the procedure of Eipper *et al.* (1983a) which, according to the authors, results in monoiodination of the peptides. On this basis, the calculated specific radioactivity of ^{125}I -D-Tyr-Val-Gly was about

Abbreviations used: PAM, peptidylglycine α -amidating mono-oxygenase; TRH, thyrotropin-releasing hormone.

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4.7 mCi/ μ g. Our early experiments demonstrated that non-enzymic amidation of D-Tyr-Val-Gly could take place, usually representing 5–10% of the enzymic reaction, but in some cases being up to approx. 30%. To correct for the interference by the non-enzymic reaction, each individual assay condition was paralleled by a control tube (blank).

Two methods were employed for determination of PAM activity. The production of D-Tyr-Val-NH₂ was analysed by radioimmunoassay, using the procedure developed by Moray *et al.* (1985). Antiserum against D-Tyr-Val-NH₂ was a gift from Dr. J. S. Kizer of the University of North Carolina School of Medicine, Chapel Hill, NC, U.S.A. Sheep anti-rabbit IgG was a gift from Dr. J. Warberg's laboratory in our institute. The reaction rate was calculated as pmol of D-Tyr-Val-NH₂ produced/h per mg of protein. In some experiments, PAM activity was measured as the turnover rate of ¹²⁵I-D-Tyr-Val-Gly to ¹²⁵I-D-Tyr-Val-NH₂. For these experiments, the reaction mixtures were applied to 1 ml SP-Sephadex columns (Eipper *et al.*, 1983a), equilibrated with 10 mM-sodium phosphate, pH 5.0. The substrate was eluted with 6 $\times$ 1.5 ml of 10 mM-sodium phosphate, pH 5.0, and product was eluted with 4 $\times$ 1.5 ml of 50 mM-sodium phosphate in 0.5 M-NaCl, pH 5.0. The turnover rate was calculated as the radioactivity in product fractions over total radioactivity in the reaction mixture. Specific activity was expressed as turnover rate/mg of protein.

Determination of protein concentration

Protein concentrations were measured by the method of Böhlen *et al.* (1973).

RESULTS

Characteristics of PAM activity in pancreatic islets

Marked PAM activity was found in islet homogenates (Fig. 1). A higher specific activity was found in soluble fractions than in membrane fractions or original homogenates. For instance, after incubation of different fractions for 2 h with about 170 000 c.p.m. of ¹²⁵I-Tyr-Val-Gly (ascorbic acid 4 mM; CuSO₄ 40 μ M), the conversion rate was 366.8%/mg of protein in the homogenate, 750.0% in the soluble fraction and 134.0% in the membrane

Fig. 1. Formation of D-Tyr-Val-NH₂ from D-Tyr-Val-Gly catalysed by homogenate of pancreatic islets from newborn rats

The homogenate was lysed by freezing and thawing, then suspended and incubated at 37 °C in a medium containing Tes (100 mM), ascorbic acid (1 mM), CuSO₄ (50 μ M), catalase (100 μ g/ml) and D-Tyr-Val-Gly (100 μ M), pH 7.0, and aerated with O₂. At different times, samples of reaction mixture were removed and the reaction stopped by freezing. The yield of D-Tyr-Val-NH₂ was analysed by radioimmunoassay immediately after incubation. Averages of duplicate assays are shown.

Fig. 2. Amidation activity of pancreatic islet homogenate from newborn rats as a function of D-Tyr-Val-Gly concentration

Incubation and assay were performed as in Fig. 1, and the incubation was for 2 h. Means $\pm$ S.E.M. of three independent experiments are presented. Inset: K_m 57.4 μ M; V_{max} 185.2 pmol/h per mg.

Fig. 3. Dependence on ascorbic acid of PAM activity in islets from newborn rats

Tissue sample preparation and incubation were as described in the legend to Fig. 1, and the incubation was for 2 h. The reaction rate was measured as pmol of D-Tyr-Val-NH₂ produced/h per mg of protein; data represent the results of four independent experiments (means $\pm$ S.E.M.).

fraction, and protein recovery was 49.8% and 42.8% in the soluble and membrane fractions respectively. The total soluble activity (specific activity $\times$ recovered protein) was much higher than the membrane-associated activity, and it even exceeded that of the homogenate.

The reaction rate was linearly dependent on incubation time within the first 2 h and then plateaued gradually (Fig. 1). The concentration-dependence curve showed that, in the presence of 1 mM-ascorbic acid and 50 μ M-CuSO₄, the V_{max} was 185.2 pmol/h per mg and the K_m was 57.4 μ M (Fig. 2).

Very low levels of PAM activity were found in exocrine tissue (< 1% of the activity found in islet tissue/mg of protein) (results not shown).

Dependence of the amidation reaction on cofactors

Ascorbic acid had a stimulating effect on amidation activity, which increased with concentration up to a certain point (Fig. 3). Treatment with ascorbate oxidase or a lack of antioxidant in the

Table 1. Specific cofactor requirements of PAM activity in pancreatic islets from newborn rats

Homogenates of pancreatic islets were incubated in 100 mM-Tes buffer containing 100 000 c.p.m. of ^{125}I -D-Tyr-Val-Gly, 2 mM-ascorbic acid, 50 μM -CuSO₄ and 100 μg of catalase/ml. The copper and catalase concentrations were as indicated in the Table. The ^{125}I -D-Tyr-Val-NH₂ content after 3 h of incubation was analysed by the method of Eipper *et al.* (1983a). The reaction rate was calculated as the percentage turnover from substrate ^{125}I -D-Tyr-Val-Gly to product ^{125}I -D-Tyr-Val-NH₂. A second experiment showed the same results.

Treatment	Relative activity (% of maximum)
Ascorbic acid in reduced form:	
Catalase ($\mu\text{g}/\text{ml}$)	
0	10.3
100	100.0
100*	12.4
200	73.4
Thiourea (0.5 mM)†	52.3
Cu ²⁺ :	
EDTA (1.0 mM)	0.6
Cu replaced with:	
Mg	0.0
Zn	1.8
Ni	0.3
Ca	0.0
O ₂ replaced with N ₂	42.8

* Medium treated with 0.5 mg of ascorbate oxidase/ml.

† No catalase.

Fig. 4. Dependence on Cu²⁺ of PAM activity in islets from newborn rats

Islets homogenate was incubated with 200 000 c.p.m. of ^{125}I -D-Tyr-Val-Gly in media containing 2 mM-ascorbic acid, 100 μg of catalase/ml and various concentrations of CuSO₄ in a Tris buffer, pH 7.0. The incubation lasted for 4 h and products were analysed using the method of Eipper *et al.* (1983a). Averages of duplicate measurements are shown.

Fig. 5. Dependence on pH of PAM activity in islets from newborn rats

Islets homogenate was suspended in media with different pH values (100 mM-Tris/HCl/base), containing 2 mM-ascorbic acid, 100 mg of catalase/ml, 40 μM -CuSO₄ and about 100 000 c.p.m. of ^{125}I -D-Tyr-Val-Gly. The incubation time was 3 h. Products were analysed using the method of Eipper *et al.* (1983a). A similar curve was obtained in a second experiment under the same conditions.

incubation medium inhibited the PAM activity significantly (Table 1).

As seen in Fig. 4, the optimum concentration of Cu²⁺ was found to be 40 μM . Replacement of Cu²⁺ with other divalent metal ions or the presence of EDTA decreased the reaction rate to the control level (Table 1). Replacing O₂ with N₂ also inhibited the amidation reaction (Table 1).

Influence of other factors

The optimum pH was 7.0 when the reaction took place in a Tris buffering system (Fig. 5). At this pH, the activity in Tris buffer was about 2–4-fold higher than in Tes buffer.

DISCUSSION

In their studies with a mitochondria/secretory granule preparation from whole pancreas tissue of rats, Ouafik *et al.* (1987) found that PAM activity was decreased by approx. 50% if the rats had been treated with streptozotocin, implying the presence of a considerable amidating activity in non- β -islet cells. In the present work, the level of PAM activity in exocrine pancreas was less than 1% of that in pancreatic islets. Since TRH, pancreastatin and pancreatic polypeptide are present in pancreatic islets, a distribution of PAM in different types of cells may occur.

In the present experiments, high PAM activity was found in a soluble phase of the homogenate. This is in accordance with findings in bovine intermediate pituitary (Eipper *et al.*, 1983a) and rat anterior pituitary (Glembotski, 1985), where the majority of PAM activity was also recovered from the soluble fraction of a granule preparation. However, in heart atrial granules from ox and rat, the activity was membrane-bound (Eipper *et al.*, 1988). The very high activity in the soluble fraction compared with the membrane fraction and homogenate indicates the presence of inhibitory factor(s) in the insoluble fractions. We do not yet know the properties and subcellular distribution of the possible inhibitor(s). We have shown that addition of a membrane suspension to the soluble fraction decreases markedly the PAM activity level in the latter (A. Zhou & N. A. Thorn, unpublished work). In rat anterior pituitary, inhibitory activity was reported to be associated with the rough endoplasmic reticulum/Golgi apparatus (Eipper *et al.*, 1983b).

To date, PAM has been characterized in a number of tissues.

Most of these enzymes show similar characteristics, such as a K_m for D-Tyr-Val-Gly between 10 and 100 μM , a V_{max} between 10 and 100 pmol/h per μg and a requirement for ascorbate (millimolar) and Cu^{2+} (micromolar) for the optimum rate of reaction. Special cases were found in porcine pituitary (Kizer *et al.*, 1986) (Cu^{2+} was not necessary and the K_m was as high as 300 μM) and rat whole pancreas (optimum $[\text{Cu}^{2+}]$ was up to 40 μM).

As to the kinetic characteristics of PAM activity, a K_m of 57.4 μM (ascorbic acid, 1 mM; CuSO_4 , 50 μM) suggests that PAM in rat islet homogenate had a similar affinity to the substrate (D-Tyr-Val-Gly) as does PAM in other tissues. For example with 1 mM-ascorbic acid and an optimum Cu^{2+} concentration, the K_m is 33 μM in a bovine intermediate pituitary granule preparation. The V_{max} for PAM in islet homogenate was 185.2 pmol/h per mg, lower than the value in all other tissues so far measured. Ouafik *et al.* (1987) reported a much higher V_{max} in their mitochondria/granule sample from whole pancreas (4 pmol/h per μg). There are multiple factors which can influence the K_m and V_{max} of enzymic reactions, including substrate, assay conditions and sample preparation. Ouafik *et al.* (1987) found that, when using <Glu-His-Pro-Gly (the precursor of TRH) as substrate, the amidation reaction was approx. 8 times slower than it was when D-Tyr-Val-Gly was used. TRH is only one of the amidated peptides in endocrine pancreas. We have no data about total contents of different peptide amides in rat islets and we have not measured PAM activity with its other natural substrates from pancreatic islet tissue.

Concerning the requirement for cofactors (ascorbate and Cu^{2+}), PAM activity in rat islet homogenate showed similar characteristics as in most other tissues, with the difference that the optimum Cu^{2+} concentration was about 10-fold higher (40 μM). The degree of purity of PAM in the preparation may be crucial for the optimum Cu^{2+} concentration. Glembotski (1985) stated that, in rat anterior pituitary, the optimum Cu^{2+} concentration for PAM was 10 times lower for a granule preparation than for homogenate. However, this seemed not to be the case in rat whole pancreas, since in a mitochondria/granule preparation the Cu^{2+} concentration was also high for optimum activity (40 μM) (Ouafik *et al.*, 1986). The PAM molecule from bovine neurointermediate pituitary has two His-rich regions, which are thought to be involved in Cu^{2+} binding (Eipper *et al.*, 1987). We do not yet know the primary structure of PAM in the pancreas. In another study on PAM cofactors in rat islets we noticed that Cu^{2+} was enriched in a granule fraction (A. Zhou, J. H. Nielsen, O. Farver & N. A. Thorn, unpublished work). Whether this implies a corresponding high Cu^{2+} -binding capacity of PAM molecules or a specialized intragranule environment needs to be explored. It should be added that Cu^{2+} may also bind to cellular proteins and/or small ligands in different subcellular fractions, possibly to a different degree in different tissues. This may result in different requirements for Cu^{2+} concentrations giving optimum PAM activity in different tissues.

It has been demonstrated in recent years that different forms of PAM exist (Stoffers & Eipper, 1990). The functional significance of this diversity is not known but tissue-specific expression of membrane and soluble forms seems to be involved. Also, it would appear that tissue-specific conditions for the concentration and regulation of cofactors may explain the differences in the activity of the enzyme(s).

The higher activity in Tris buffer than in Tes buffer at pH 7.0 has not been reported in other studies. The mechanism of this phenomenon is obscure.

These studies were supported by the Danish Medical Research Council, the Danish Space Research Committee, the Danish Government Biotechnology and FTU Programs, the Danish Association against Multiple Sclerosis, the Haensch, Ib Henriksen, Nordisk Insulin, the Alfred Benzon Foundation, the Foundation of December 17 1981 and the Foundation for Advancement of Medical Science. Benny Welinder of the Hagedorn Research Laboratory performed the h.p.l.c. analysis of the purchased peptides for PAM assay. The excellent technical assistance of Birthe Lynderup Christensen and Inge Kjeldsen is gratefully acknowledged. We thank Dr. Carl Jørgen Hedeskov for his valuable suggestions and comments on the manuscript.

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