FEBS 22323 FEBS Letters 455 (1999) 159–161

Evidence for tissue-specific forms of glutaminyl cyclase

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Received 13 April 1999; received in revised form 16 June 1999

Abstract Glutaminyl cyclase (QC) is responsible for the presence of pyroglutamyl residues in many neuroendocrine peptides. An examination of the bovine tissue distribution of QC immunoreactivity, enzyme activity, and mRNA confirmed that QC was abundant in brain and pituitary by all three measures. However, enzymatic activity was considerably more widespread than either immunoreactivity or mRNA, suggesting multiple enzyme forms. Partially purified QC from bovine spleen differed significantly from the known bovine pituitary QC in physical and catalytic properties. We propose that this form of glutaminyl cyclase plays a role in the posttranslational processing of constitutively secreted pGlu-containing proteins.

Key words: Glutamine; Pyroglutamic acid; Secretory component; Spleen

1. Introduction

Mammals contain numerous secretory peptides and proteins possessing an amino-terminal pyroglutamyl (pGlu) residue. Hormones such as the thyrotropin-releasing hormone and gonadotropin-releasing hormone, and neurotransmitters such as neurotensin are representative peptides whose amino-terminus is blocked by this residue. This posttranslational modification is catalyzed in vivo by glutaminyl cyclase (QC, EC 2.3.2.5) [1,2] which acts upon the glutaminyl residues unmasked by prohormone convertase cleavage of precursor peptides [3]. Subcellular distribution studies have confirmed the presence of glutaminyl cyclase in pituitary secretory granules [4] and cytochemical studies in anterior pituitary have localized QC protein and mRNA exclusively to somatotrophs [5]. The bovine and human pituitary QC cDNA have been sequenced and found to encode a 361 amino acid protein with a 27 amino acid endoplasmic reticulum signal [6,7]. Northern blotting of mRNA derived from a variety of bovine tissues revealed a single 2.1 kb species of QC mRNA in all reactive tissues [6] with high levels in brain tissues.

The expression of spleen QC was not initially detected in the Northern blot by Spiess and coworkers [6]. However, because of the presence of the pyroglutamyl group in IgG, those authors speculated that spleen may contain an alternative form of QC. This suggestion was strengthened by the report by Kizer and coworkers [1] of QC in rat B lymphocytes. It was the purpose of this study to examine the possibility of multiple forms of QC in bovine tissues and, in particular, to determine if spleen indeed contains a form of glutaminyl cyclase which differs from that found in pituitary.

2. Materials and methods

2.1. Tissue distribution

For the bovine tissue distribution the tissues were extracted in the presence of protease inhibitors and separated into soluble and membrane fractions by the method of May et al. [8]. Protein was determined with the Coomassie Plus reagent from Pierce using bovine serum albumin as a standard. Enzymatic activity of tissue extracts was determined with the dansylated pre-neurotensin fragment HPLC assay of Consalvo et al. [9]. Western blotting was performed with antibodies raised against an amino-terminal peptide derived from the bovine pituitary QC sequence [7].

2.2. Partial purification and characterization of bovine spleen glutaminyl cyclase

Twenty grams of tissue was removed from a frozen mature bovine spleen and minced with a razor blade. The tissue sample was homogenized in a Waring blender with five volumes of chilled 0.02 M potassium phosphate buffer, pH 6.5, subjected to two freeze/thaw cycles, and centrifuged for 30 min at $36\,300\times g$. The supernatant pH was lowered to 5 via the dropwise addition of 0.1 M acetic acid; the solution was stirred for an additional 15 min, and then centrifuged as before. The pH of the supernatant pool was brought to 7.0 by dropwise addition of 1 M K_3PO_4. The extract was then fractionated with ammonium sulfate and the 50–65% fraction retained for dialysis against 0.02 M MOPS, pH 7.2, containing 0.1 M NaCl and 5% glycerol. The dialysate was clarified by centrifugation and diluted five fold with 0.02 M MOPS, pH 7.2, immediately prior to chromatography on a DE52 cellulose (Whatman) column (1.5×10 cm) equilibrated with the same buffer. The column was washed with five column volumes of equilibration buffer and eluted with a 20 column volume salt gradient from 0 M to 0.5 M NaCl. Active fractions were pooled, precipitated with 70% ammonium sulfate, and dialyzed as before. The purified enzyme passed unhindered through both concanavalin A Sepharose and lentil lectin Sepharose columns at pH 7.2 without even residual activity eluted with methyl mannose. Gel filtration chromatography of the purified spleen preparation was accomplished with a Sephacryl S-200 column (1.5×40 cm) calibrated with bovine serum albumin, horseradish peroxidase, carbonic anhydrase and myoglobin.

Enzymatic activity was measured during spleen QC purification and characterization by a modification of the method of Bateman [10] in which the buffer was 0.1 M sodium borate, pH 8.5, and the substrate was glutaminamide (Bachem). For the substrate specificity study Gln-X peptides (Bachem) were substituted for glutaminamide.

3. Results

Fig. 1 shows that the initial tissue distribution study of QC mRNA by Pohl and coworkers [6] correlated relatively well with Western blot and enzyme activity analysis of the various brain tissues. The Western blot (Fig. 2) showed two primary immunoreactive species, a 40 kDa protein found exclusively in the soluble pituitary extract and a 32 kDa band found in the soluble fraction of all tissues which showed immunoreactivity.

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PII: S0014-5793(99)00872-8

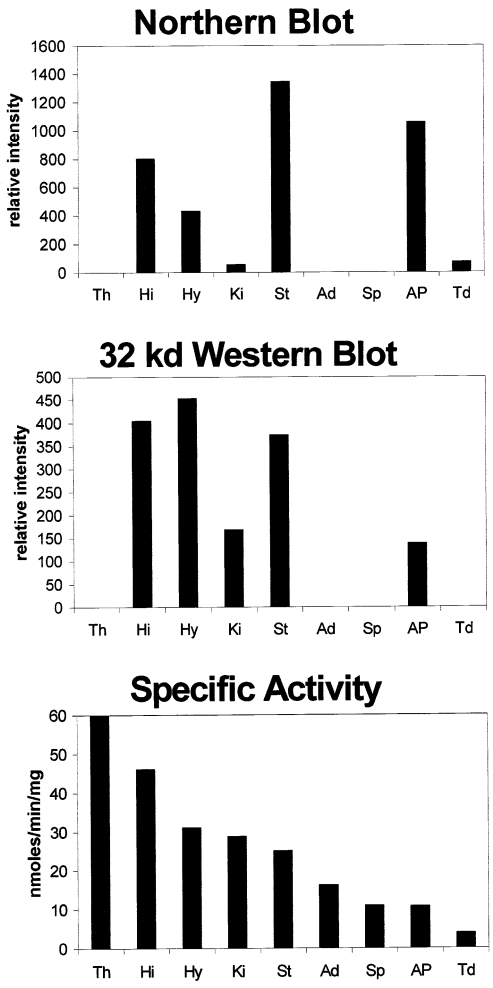

Fig. 1. Distribution of glutaminyl cyclase in bovine tissues. Comparison of Northern blot, Western blot and enzyme-specific activity. Lanes are Th-thymus, Hi-hippocampus, Hy-hypothalamus, Ki-kidney, St-striatum, Ad-adrenal, Sp-spleen, AP-anterior pituitary, Td-thyroid. Specific activity measurements are the means of at least four determinations with an error of less than 18% in all lanes.

Although the larger pituitary QC band is intense, the specific activity of the pituitary extract is surprisingly low in comparison to the brain extracts. This may indicate a form of activation of QC via proteolytic processing. The antibody is directed towards the amino-terminus, suggesting that the difference in the two immunoreactive proteins lies in proteolytic cleavage within a hydrophilic region approximately 80 amino acids from the carboxy-terminus which probably represents an exposed surface loop.

Table 1
Substrate specificity for partially purified spleen QC at pH 8.5 and 37°C

Substrate	Specific activity (nmol/min/mg)	Relative activity (%)
Gln-NH_2	28.5 ± 0.6	100.0
Gln-Gln-Gln	16.4 ± 0.3	57.5
Gln-Gly	12.4 ± 0.3	43.5
Gln-Gly-Pro	11.4 ± 0.6	40.0
Gln-Ala	10.6 ± 0.8	37.2
Gln-Glu	9.3 ± 0.5	32.6
Gln-Gln	6.4 ± 0.6	22.5
D-Gln	Negligible	–
L-Gln	Negligible	–

Substrate concentration was 3 mM ($n = 3$, mean ± S.D.). Unless otherwise noted, all amino acids are the L isomers.

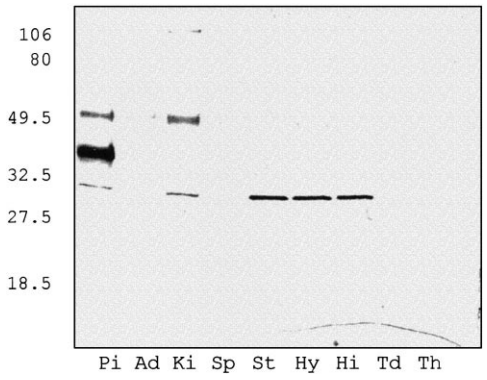

Fig. 2. Western blot analysis of bovine tissue soluble extracts. Lanes are Pi-pituitary, Ad-adrenal, Ki-kidney, Sp-spleen, St-striatum, Hy-hypothalamus, Hi-hippocampus, Td-thyroid, Th-thymus. Four micrograms of protein were loaded in each lane. The 50 kDa band in lanes Pi and Ki was also observed in preimmune controls and is presumed to be an artifact.

Further examination of Fig. 1 reveals marked discrepancies between the three measures of expression. For example, spleen showed neither immunoreactivity nor mRNA expression even though the spleen extract contained enzymatic specific activity approximately equivalent to that of pituitary. Fractionation of the spleen QC activity with ammonium sulfate at 0°C revealed that the activity remained soluble until the saturation level reached 50% while pituitary QC precipitated at 30% saturation [9]. Anion exchange chromatography following ammonium sulfate precipitation resulted in the elution of enzyme activity in a single peak at a salt concentration of approximately 0.22 M. The resulting enzyme preparation was highly active and stable at 4°C for several weeks. Gel filtration of the concentrated pool yielded a peak of activity corresponding to a molecular weight of 48 500 Da. This is notably higher than the 38 000 Da reported for the bovine pituitary enzyme [4,6].

Testing of the purified spleen QC action upon a series of

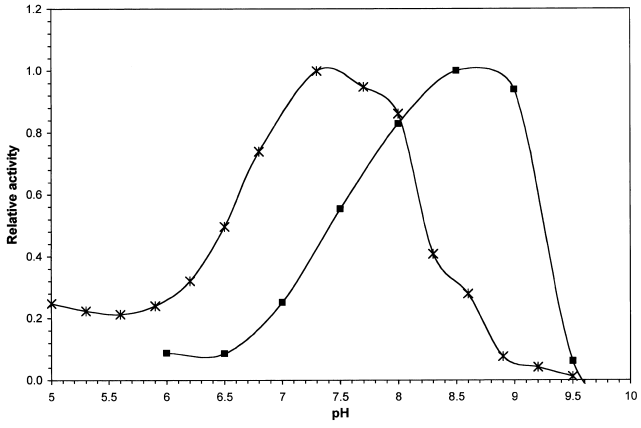

Fig. 3. Dependence of enzymatic activity of bovine spleen QC (squares) and recombinant human pituitary QC [22] (asterisks) on pH. Activities were normalized for comparison. Glutaminamide (5 mM) was the substrate and MOPS (6.0–8.0), EPPS (7.0–9.0) and borate (8.0–10.0) were substituted for the buffer in the standard protocol [10].

Gln-X peptides (Table 1) revealed that glutaminamide was the optimal substrate. In contrast to the pituitary enzyme [7,10], Gln-Gln proved to be the least effective substrate. As expected, neither of the free amino acid isomers of glutamine showed detectable reaction. Spleen QC exhibited maximal enzyme activity at pH 8.5, an alkaline shift of one unit when compared to the pH optimum of the pituitary enzyme (Fig. 3).

4. Discussion

The tissue distribution of QC enzymatic activity indicated the presence of QC in every tissue examined. As predicted by the lack of a membrane-anchoring domain in the known amino acid sequence of glutaminyl cyclase, enzyme activity and immunoreactivity were exclusively found in the soluble fractions of the tissue extracts. This is in contrast to the numerous membrane-bound forms of another peptide terminus-modifying enzyme, peptidyl-glycine α-amidating monooxygenase (PAM) [8]. Likewise, thymus and spleen exhibited high QC-specific activity whereas these tissues contain little, if any, PAM activity or mRNA [11–13]. These observations are consistent with the absence of known amidated peptides produced by thymus and spleen as well as the presence of several proteins of the immune system which contain the pyroglutamyl group including both the heavy [14,15] and light chains [16] of immunoglobulins; the J chains of polymeric IgA and IgM [17]; the C1q subcomponent of the classical complement pathway [18]; and human interferon [19].

Because of the lack of pituitary QC mRNA and immunoreactivity in spleen and the presence of the pyroglutamyl residue on proteins rather than peptides in this tissue, we decided to examine the glutaminyl peptide cyclizing activity in spleen. Partial purification of QC activity from soluble spleen extracts yielded an enzyme fraction with a specific activity comparable to the pituitary glutaminyl cyclase after anion exchange chromatography [10]. The partially purified enzyme was stable upon storage at 4°C and appeared to differ from its pituitary counterpart in terms of size, pH optimum for activity, substrate specificity, and solubility in ammonium sulfate. The inability of the spleen QC to adsorb to immobilized lectins, also in contrast to reports of pituitary QC [1], suggested that it is not highly glycosylated. A final biochemical difference between the spleen and pituitary enzymes is the lack of discernible inhibition of the spleen QC by imidazole at concentrations considerably higher than those required for inhibition of pituitary QC (J.S. Temple and R.C. Bateman, Jr., manuscript in preparation). In sum, spleen and pituitary QC appear to have distinctly different physical and catalytic properties.

The genetic origin of the various QC forms in unclear. Other neuroendocrine biosynthetic enzymes such as PAM [20] and dopamine β-hydroxylase [21] are expressed as tissue-specific forms derived from a single gene. It is quite possible that all bovine glutaminyl cyclases are derived also from a single gene and, in fact, a single pituitary QC gene has been localized to human chromosome 2 (RH map A002E27; UniGene Hs. 79033). However, another very real possibility is that the spleen QC is the product of a second gene of low homology to the one encoding the pituitary enzyme. This would be consistent with the absence of spleen QC mRNA in the Northern blot and our inability in multiple attempts to amplify spleen QC cDNA using RT-PCR and primers which consistently work well with mRNA from pituitary and hypothalamus [7,22]. The solution of this mystery will have to await complete purification and peptide sequencing of the spleen QC.

The presence of several constitutively expressed pGlu-containing proteins in the spleen indicates a need for glutaminyl cyclase activity in the constitutive secretory pathway. The enzyme reported here is a likely candidate for this role as evidenced by its relative abundance in spleen and a pH activity optimum which makes its action in the acidic environment of a neuroendocrine secretory granule difficult to envision. Also, as evidenced by its solubility in the acid precipitation step of the purification procedure, spleen glutaminyl cyclase does not aggregate at low pH like proteins directed to the regulated secretory pathway [23,24]. It appears likely, therefore, that the role of this novel form of QC is to catalyze pyroglutamyl residue formation at the amino-terminus of proteins secreted through the constitutive pathway.

Acknowledgements: This work was supported by NIH Grant DK41892 to R.C.B., the NSF EPSCOR Program, the state of Mississippi and the University of Southern Mississippi.

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