INSULIN DEGRADATION IN INSULINOMA: EVIDENCE FOR THE OCCURRENCE OF AN INACTIVE FORM OF GLUTATHIONE-INSULIN TRANSHYDROGENASE AND FOR THE ABSENCE OF INSULIN A AND B CHAINS DEGRADING PROTEASE(S). *

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Summary: Islet cell tumors (insulinomas) have been found to contain insulin-degrading activity. Apparent K_m values for insulin obtained with tumor extracts were similar to those found for other tissues and for purified glutathione-insulin transhydrogenase (GIT). In Ouchterlony double diffusion experiments with antibody to purified human liver GIT, each tumor extract gave a single precipitation band of identity with purified human liver GIT. Examination by chromatography on Sephadex G-75 of the products formed from ^{125}I-insulin upon incubation with tumor extracts showed the same products (A chain, and B chain rich-A chain aggregate) as previously found with purified GIT; however, there was no further degradation (i.e., proteolysis) of A chain to low molecular weight components. These results indicate that the insulin-degrading activity present in the islet tumor is, in fact, GIT and that the protease(s) that further catabolizes the insulin A and B chains is apparently missing in the insulinoma. These data could be interpreted to indicate that the function of GIT in this tissue is to promote the biosynthesis of proinsulin and insulin rather than their degradation. Data are also presented which indicate that in insulinoma GIT is present in an inactive state as a divalent metal ion complex since it could be activated with EDTA and/or GSH.

Recent work from this laboratory has (1-3) shown that insulin is metabolized in a sequential manner: first, the hormone is split at the disulfide bonds under the action of glutathione-insulin transhydrogenase (GIT, thiol: protein disulfide oxidoreductase, EC 1.8.4.2) with the formation of A and B chains, and second, the resulting polypeptide chains are further catabolized by proteolysis. This sequential pathway has been demonstrated in all the rat tissues examined so far

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(liver, kidney, heart, skeletal muscle and adipose tissue). With liver (3) the sequential pathway has been demonstrated to function both at physiologic (10^{-9}M) as well as pharmacologic (10^{-5}M) concentrations of insulin. In the liver GIT occurs primarily in the microsomal fraction while the A and B chains degrading protease(s) is located mainly in the high speed supernatant fraction (4). Because GIT occurs ubiquitously (5), we suggested previously (2) that the sequential mode of insulin degradation is probably as widely distributed as is GIT. The presence of insulin-degrading activity in isolated rat islets has been reported by others (6, 7); attempts in this laboratory to demonstrate such an activity in the islet gave equivocal results. Recently we had an opportunity to study insulin degradation in insulinoma tissues. We have made two new heretofore unknown observations about this enzyme system. In insulinoma tissues, contrary to our previous findings in other tissues the second proteolytic system of insulin degradative pathway (i.e., the protease(s) that catalyzes hydrolysis of insulin A and B chains) is apparently absent and the GIT is apparently present in an almost totally inactive state, probably as a divalent metal ion complex. This is the first tissue found in which GIT is present in an inactive form and which has the second enzyme system of insulin degradation missing.

Methods: Insulinoma tissues (fresh frozen) from two adult patients with a history of spontaneous hypoglycemia were used. All the procedures used have been described in our previous publications. The references, with pertinent details, are identified at appropriate places and in the legends to the figures and table. The tissue extracts were prepared by homogenizing 0.4 gm of tumor tissue in 2 ml of 0.25 M sucrose-50mM tris buffer, pH 7.5 with a Polytron homogenizer (1), and centrifuging at 5100g for 10 min and 10000g for 15 min; the sediment was discarded after each centrifugation. All experiments reported here were carried out using this tissue extract.

Results and Discussion: Experiments were carried out using our usual incubation medium (0.1 M potassium phosphate-5 mM EDTA, pH 7.5). Both tumor extracts were found to be capable of degrading insulin (Figure 1). The degrading activity in both extracts was increased by the addition of 1 mM GSH; the increase in the specific activity was 1.5-fold in the case of extract J.C. and 3.5-fold with extract H.T. when insulin was present at 1 μM.The apparent specific activity in the presence of GSH was about 13 times greater in the extract H.T. (1968 units/mg protein) than that in the extract J.C. (156 units/mg protein), while the

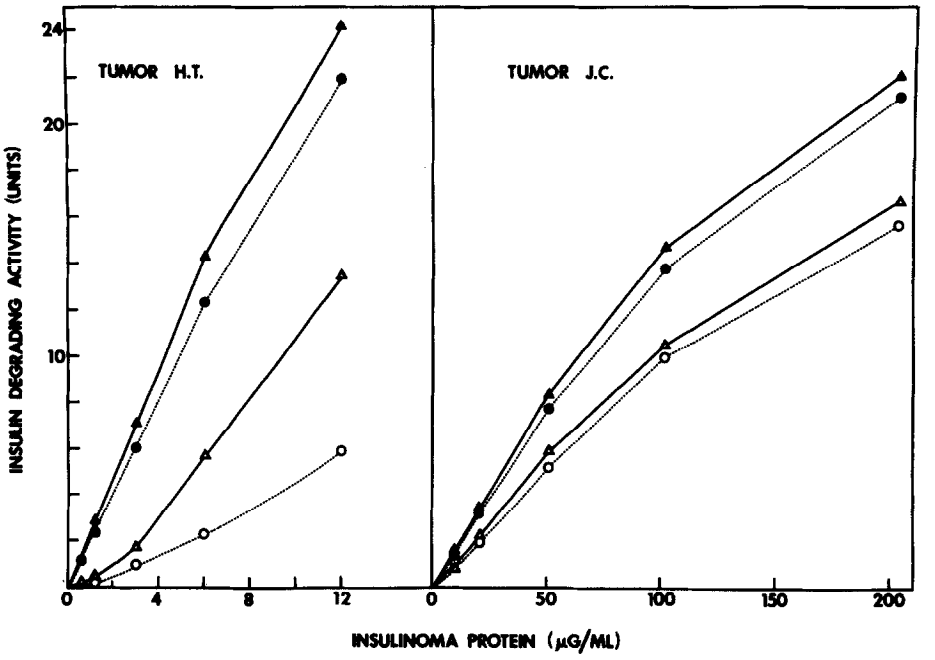

Figure 1. Insulin degradation as a function of the amount of insulinoma protein. A mixture of ^{125}I-labelled insulin and unlabelled insulin was incubated at 37° in a total volume of 1 ml of 0.1 M potassium phosphate-5 mM EDTA-0.3% bovine serum albumin (final pH 7.5) with several levels of insulinoma protein (see ref. 5, 16, 17). After five minutes, the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid . The precipitates were collected by centrifugation. The rates of insulin degradation shown have been corrected for degradation occuring in matched control incubations with tissue protein omitted. One unit is arbitrarily defined as the net degradation of one percent of the insulin present in 5 minutes under the conditions described. 10 nM insulin, △——△ ; 10 nM insulin + 1 mM GSH, ▲——▲ ; 1 μM insulin, O·····O ; 1 μM insulin + 1 mM GSH, ●·····● .

immunoreactive insulin + proinsulin content was about 150 times greater in the extract J.C. (25.5 μg insulin/mg of protein) than in the extract H.T. (0.17 μg insulin/mg of protein). After accounting for these differences in the substrate (insulin + proinsulin)content by determining the values of V_m per mg of protein (i.e., by extrapolating to infinite insulin concentration), the insulin-degrading activity was 7 times greater in the extract H.T.

Two lines of evidence demonstrate that the insulin-degrading activity in the insulinoma is, in fact, GIT. Apparent K_m values for insulin were determined by using ten different insulin concentrations from 0.0083 to 33.4 μM in the presence of 1 mM GSH. The K_m values for insulin derived by a direct nonlinear regression fit of the Michaelis-Menten equation were 4.0 $\pm$ 0.36 μM for H.T. and 7.8 $\pm$ 0.72 μM for J.C. These values are in the same range as those found for rat tissues and for purified GIT using the same assay procedure (5). Unequivocal evidence for the presence of GIT in the tumor extracts was provided by double immunodiffusion experiments (Figure 2) with antibody to purified human liver GIT: a precipitin band in a similar position and continuous with the precipitin band of pure human liver GIT was obtained with each tumor extract.

Insulinoma tissue extracts were subjected to techniques previously used (1-3) to determine whether insulin degradation in this tissue also takes place via the two-step sequential pathway. In this technique, ^{125}I-insulin is incubated (in buffers containing GSH, no GSH, EDTA, and no EDTA) with a tissue extract for varying times and the products of insulin degradation are examined by chromatography on Sephadex G-75. With all tissues studied previously, there is a transient accumulation of an intermediate product (A chain) which is further degraded by protease(s) to low molecular weight components. The

amount and rate of accumulation of A chain are dependent upon the relative concentrations of tissue (and the relative amounts of two enzyme systems, i.e., GIT and insulin A/B chains degrading protease(s) present in a given amount of tissue), insulin, thiol donors (endogenous plus exogenous), and the type of incubation medium. There is a greater accumulation of A chain in the presence of EDTA, since it inhibits the second enzyme system. The results presented in Table 1 show that there is little or no conversion of A chain to low mol. wt. components under any conditions, even when almost all the insulin has been transformed to A chain and B chain (or B chain rich-A chain aggregate). Thus the protease(s) that degrades A and B chains is apparently absent in the insulinoma tissue. Incubations which contained neither EDTA nor GSH gave

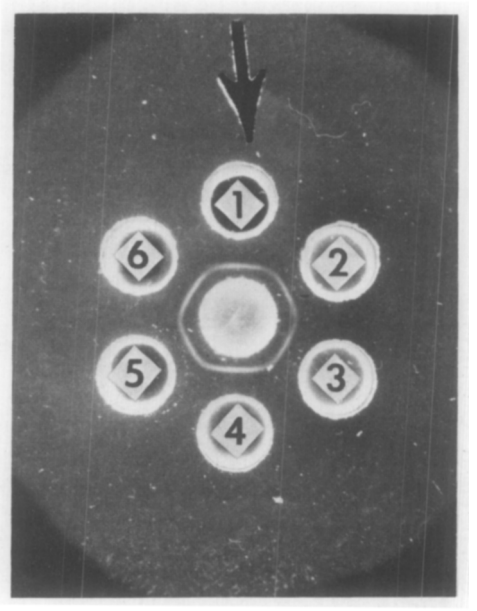

Figure 2. Ouchterlony double immunodiffusion of insulinoma extracts with rabbit antiserum to purified human liver GIT (16, 18). The center well contained 50 μl antiserum. Each of the peripheral wells received 50 μl of: (1) purified human liver GIT, 6 μg; (2) tumor extract J.C., 1.5 mg (116 units); (3) tumor extract J.C., 1.25 mg (96 units); (4) tumor extract H.T., 0.15 mg (297 units); (5) tumor extract H.T., 0.05 mg (99 units); (6) tumor extract H.T., 0.10 mg (198 units). Diffusion was carried out for forty hours at 5° C.

Table I

Results of Chromatographic Analysis of Insulin Degradation Products. ^{125}I-insulin and unlabelled insulin (1 μM) was incubated at 37° in 0.1 M potassium phosphate buffer (pH 7.5) containing 0.3% bovine serum albumin using the experimental conditions shown below in the table. Incubations were terminated by the addition of N-ethylmaleimide solution (15 mM final concentration). After the addition of solid urea (1.44 g) and 40 μl of a mixture of unlabelled S-sulfonated A chain (40 μg) and B chain (60 μg) as carrier, the volume of the incubation mixture was brought to 6 ml with glacial acetic acid and the mixture was chromatographed on a 2 x 45 cm. Sephadex G-75 column with 50% acetic acid as eluent. See references 1-3 for complete details. The ^{125}I-insulin used contained 90% of the radioactivity of the original insulin in A chain and 10% in B chain.

Experimental Conditions					% of Radioactivity in			
Expt. No.	EDTA 5 mM	GSH 1mM	Tumor protein mg/ml	Incubation Period min.	Insulin	B chain rich-A chain aggregate	A chain	Low Mol. wt. components
		Islet Tumor H.T.						
1	+	-	0.02	10	93	5	2	0
2	+	-	0.10	10	40	8	52	0
3	+	-	0.20	10	15	7	78	0
4	+	-	0	10	100	0	0	0
5	-	-	0.20	10	90	8	2	0
6	-	+	0.20	10	19	14	67	0
7	-	+	0	10	90	6	4	0
8	-	+	0.2	60	1	13	79	7
9	-	+	0	60	65	8	25	2
10	-	+	0.2*	10	3	6	91	0
11	-	-	0.2*	60	64	7	28	1
12	-	+	0.2*	60	1	8	90	1
		Islet Tumor J.C.						
13	-	-	2.04	60	77	13	10	0
14	-	+	2.04	60	10	16	71	3
15	+	-	2.04	60	34	16	49	1
16	+	+	2.04	60	4	18	78	0
17	-	+	0	60	72	13	12	3

* In order to remove the divalent metals, tumor extract for these experiments was pretreated with 25 mM EDTA and then dialyzed extensively vs. phosphate buffer.

almost no disappearance of insulin (experiments 5 and 13 in the table), indicating that in the insulinoma GIT apparently occurs in an almost totally inactive state. This lack of activity is probably because of the occurrence of a divalent metal ion complex since the GIT could be activated by EDTA and/or GSH. Experiments to determine the mechanisms that bring about the activation of the inactive GIT complex are in progress in this laboratory.

In addition to inactivation of insulin, GIT has been found to promote the activation of reduced or "scrambled" forms of insulin (8, 9), proinsulin (10) and ribonuclease (4, 11). The absence of the second enzyme system in insulinoma could be interpreted to indicate that the function of GIT in this tissue is, as might be expected, to promote the synthesis of proinsulin and insulin (and possibly other disulfide-containing proteins) rather than their degradation. However, it remains to be determined whether the A/B chains degrading protease(s) is absent in normal β cells or is present in β cells but lost in the transformed insulinoma cell; and if the latter is true, whether this loss is characteristic only of the insulinoma cell or is a general characteristic of all tumor cells.

Three mechanisms controlling functional GIT have been recognized previously: (a) Liver GIT in the rat is under feedback control by blood insulin (12-14); there are adaptive changes in the amount of this enzyme in response to starvation and diabetes and these changes correlate with the circulating level of insulin. (b) The enzyme appears to be synthesized and stored in endoplasmic reticulum (4) and a small amount is translocated via membrane biogenesis to plasma (cell) membrane (15) possibly in response to physiological needs. (c) A major part (~80%) of the microsomal enzyme occurs in a latent state as a phospholipoprotein complex and is released by agents such as phospholipases A and C (4); such release mechanisms probably regulate

the effective levels of GIT. The findings in the current study of the occurrence of an inactive form of the enzyme (as a divalent metal ion complex) suggest the existence of an additional control mechanism. Thus, there appears to be several controls over GIT.

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