Biochimica et Biophysica Acta, 422 (1976) 254—259

BBA 67711

DEMONSTRATION OF INSULIN DEGRADATION BY THIOL-PROTEIN DISULFIDE OXIDOREDUCTASE (GLUTATHIONE-INSULIN TRANSHYDROGENASE) AND PROTEINASES OF PANCREATIC ISLETS *

K -D KOHNERT, H JAHR, S SCHMIDT, H -J HAHN and H ZUHLKE

Zentralinstitut fur Diabetes "Gerhardt Katsch", Bereich experimentelle Diabetesforschung, 2201 Karlsburg (G D R)

(Received September 2nd, 1975)

Summary

Homogenate preparations of pancreatic islets have been found to degrade insulin by cleavage of the interchain disulfide bonds, followed by proteolysis of the resulting A and B chains A proteolytic system of the pancreatic islets splitting not only ^{125}I-labeled insulin A chain but also ^{125}I-labeled glucagon at pH 7.0, was shown to be activated by glutathione and inhibited by EDTA The results suggest that pancreatic islets contain both the thiol-protein disulfide oxidoreductase (glutathione protein-disulfide oxidoreductase, EC 1 8 4 2) and the A and B chain-degrading enzyme(s) The effects of EDTA argue against the implication of cathepsins in insulin breakdown under the experimental conditions employed

Introduction

Studies on explants of pancreas [1] have shown that intracellular insulin catabolism seems to play an important part in regulating the insulin content of islets of Langerhans The mechanisms and the enzymes involved in the degradation process have not yet been elucidated. The detection of insulin A and B peptide chains in islets [2,3] raised the question as to whether insulin destruction takes place in a step-wise manner as first reported by Varandani [4,5] for several other tissue preparations In this paper, evidence is presented that insulin degradation in homogenized islets is catalyzed by the action of thiol-protein disulfide oxidoreductase and proteinases splitting the separated insulin chains

* This work was presented in part at the 9th FEBS Meeting Budapest (Hungary), August 1974

Materials and Methods

Chemicals

Bovine insulin chromatographically purified [6] was obtained from VEB Berlin-Chemie (G.D R) and crystallized glucagon was from Eli Lilly GmbH Giessen (G F R) Insulin and glucagon labeled with ^{125}I were prepared by the ICl method [7] and a modified Hunter and Greenwood procedure, respectively [8]. The radioiodinated insulin preparation used contained less than 0 75 atoms of iodine and the glucagon preparation 1 0 atoms of iodine per molecule. S-sulfonated insulin A chain was prepared by sulfitolysis of ^{125}I-labeled insulin [9] Glutathione was purchased from Merck AG Darmstadt (G F.R) and collagenase (CLS 2 FP, 142 units/mg) from Biochemical Corp Worthington Freehold, N J (U S A) All other chemicals were of reagent grade and were obtained commercially.

Isolation of pancreatic islets and preparation of tissue homogenates

Islets of Langerhans were isolated by collagenase digestion [10] of the pancreata of 12 h-fasted Wistar rats (150—200 g body weight) as described [11]. Only islets free from contamination by acinar tissue were picked up with modified Pasteur micropipettes under a stereo microscope and washed by eight transfers to fresh Hanks' solution

Batches of 500—600 islets were homogenized at 4° C in 120 µl distilled water by means of a glass homogenizer fitted with a Teflon pestle and used after freezing at −20° C and thawing (1×) for the detection of enzymatic activity The micro-method of Lowry et al [12] was used for protein determination

Assay of enzymatic activity

20 µl of homogenized islets were incubated after 2 min of preincubation with glucagon containing tracer amounts of ^{125}I-labeled glucagon in 0 1 M potassium phosphate buffer, with the additions described in the legend of Fig 2 Control experiments contained 0 1—1 0 µg of collagenase per incubation in place of islet homogenate Collagenolytic activity was determined using 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine (Serva, Heidelberg, G. F R) according to Wunsch and Heidrich [13] α-Amylase activity was measured as described by Bernfeld [14] All incubations were carried out in closed plastic tubes (Netheler and Hinz, Hamburg-Eppendorf, G.F R.)

Results

In order to study the manner of degradation, 15 µg of insulin containing a tracer amount of ^{125}I-labeled insulin was incubated in 1 mM glutathione with 46 µg of islet protein for various times and chromatographed on Sephadex G-75. Some representative profiles of radioactivity obtained upon incubation with islet homogenate preparations in 0.1 M potassium phosphate containing 5 mM EDTA as well as in Krebs-Ringer-bicarbonate are shown in Fig. 1. In the EDTA-containing buffer, as the radioactivity in the insulin peak (Fractions 20—34) disappeared, two radioactive peaks, eluted at the position of sulfonated A chain and at the void volume, were formed. After 180 min of incubation

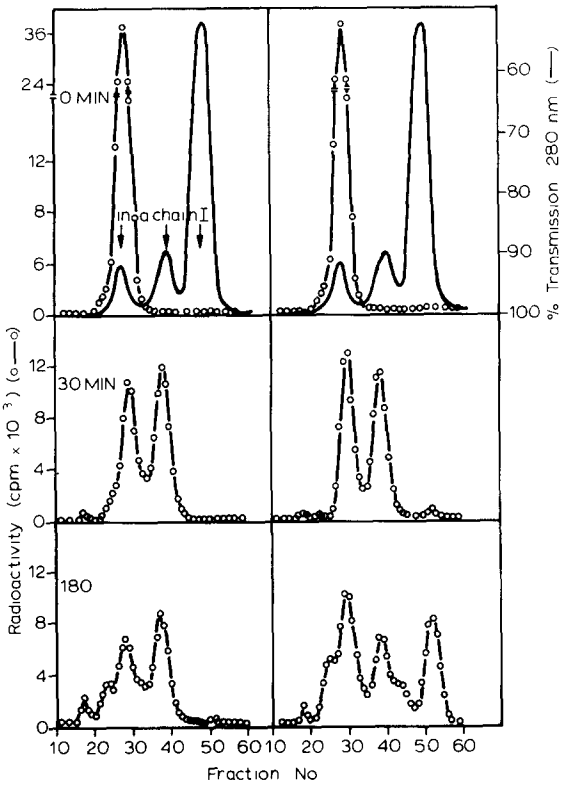

Fig 1 Gel chromatography pattern of insulin (15 μg) containing a tracer amount of ^{125}I-labeled insulin that had been exposed to homogenate of isolated rat pancreatic islets (46 μg protein per incubation) either in 1 mM glutathione, 0 1 M potassium phosphate/5 mM EDTA buffer (pH 7 0) (left) or 1 mM glutathione, Krebs-Ringer bicarbonate buffer (1 2 mM KH_2PO_4, 115 mM NaCl, 4 65 mM KCl, 2 56 mM $CaCl_2$, 1 2 mM $MgSO_4$ $7H_2O$, 24 mM $NaHCO_3$, pH 7 0) (right) at 37°C for the indicated time periods Incubations were terminated by cooling the samples in ice and by subsequent addition of 20 μl of 0 1 M *N*-ethylmaleimide and 20 μl of glacial acetic acid 100 μl of the entire mixture (120 μl) were applied to a Sephadex G-75 column (1 cm X 50 cm), equilibrated and eluted with 50% acetic acid at a flow rate of 8 5 ml/h 1 ml fractions were collected and counted for radioactivity in a Packard Auto-Gamma Counter Radioactivity in cpm, o———o, % transmission at 280 nm, ——— Vertical arrows denote from left to right, the insulin peak (in), the peak of S-sulfonated A chain and the peak of low molecular weight components (I^-) consisting of salt and *N*-ethylmaleimide.

almost no radioactivity appeared in the low molecular weight components, whereas in the case of incubation in Krebs buffer, one major radioactive peak appeared at the position of low molecular weight components Preincubation of the homogenate preparations with glutathione for 5 min instead of the usual 2 min and subsequent treatment for the same time with 1 mM iodoacetate or iodoacetamide produced complete inhibition of the insulin-degrading activity even in the presence of 3 mM glutathione

In order to establish the identity of the material in the peak eluting at the position of A chain, the fractions of this peak were pooled, lyophilized, mixed with unlabeled S-sulfonated A chain, and rechromatographed, the radioactive peak coinciding with the ultraviolet-absorbing peak When in separate experiments ^{125}I-labeled A chain was exposed to homogenate preparations of pancreatic islets in Krebs-Ringer-bicarbonate containing 1 mM glutathione, essen-

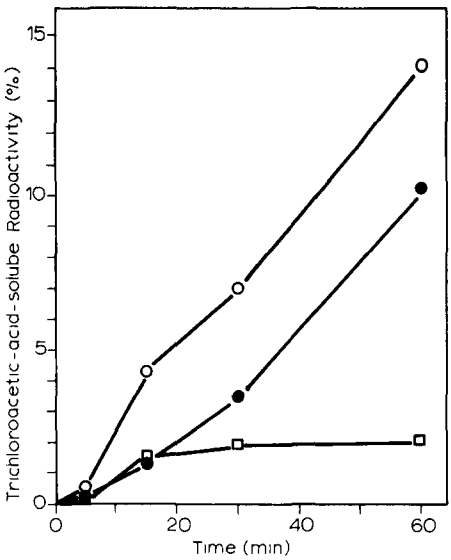

Fig 2 Time course of glucagon degradation by homogenate preparations of rat pancreatic islets For each determination 20 μl of homogenate (12 μg of tissue protein) were preincubated for 2 min in 200 μl of 1 88 mM glutathione, 0 1 M potassium phosphate buffer, ○———○, 0 1 M potassium phosphate buffer, ●———●, and 1 88 mM glutathione, 0 1 M potassium phosphate/5 mM EDTA buffer, □———□ After this time, 100 μl of glucagon solution (1 25 μg) containing a tracer amount of ^{125}I-labeled glucagon was added and the whole mixture incubated for the time given The reaction was terminated by cooling the samples in an ice-bath for 2 min, followed by subsequent addition of 100 μl 2% bovine serum albumin and 200 μl of ice-cold 15% trichloroacetic acid 500 μl of the resulting supernatants were assayed for radioactivity All incubations were performed at pH 7 0 at 37°C and the values shown are triplicate determinations from a series of representative experiments

tially the same events regarding the degradation of the A chain were obtained as shown in Fig. 1 (right).

In addition, experiments were carried out in which glucagon containing tracer amounts of ^{125}I-labeled glucagon was incubated with pancreatic islet homogenate for various times in phosphate buffer (Fig 2) Again, the degrada-

TABLE I

ACTIVITY OF α-AYMLASE IN THE HOMOGENATE PREPARATIONS OF ISOLATED RAT PANCREATIC ISLETS

Determination of activity was performed according to Bernfeld [14] by incubation of 10 μl islet homogenate (about 10 μg protein) with 200 μl soluble starch for 5 min at 20°C The reaction was interrupted by addition of 200 μl 3,5-dinitrosalicylic acid After boiling for 5 min, cooling and dilution, the reduction product formed was determined photometrically

Islet preparation batch No	Amylase activity	
	% *	mU/μg protein **
1	4	4 4
2	2	2 2
3	<1	<1

* Amylase activity is expressed in percent of that of the whole pancreas

** D-(+)-maltose was used as standard

tion of this peptide was activated by glutathione and inhibited in the presence of EDTA, indicating that this proteolytic system in the islets of Langerhans is not capable of degrading insulin A chains only, but other peptides also The presence of the chymotrypsin inhibitor Tos-PheCH$_2$ Cl (0 5 mM) in the incubation medium had no effect on the degrading activity To evaluate possible interference of nonspecific proteolytic activities from the collagenase employed in the isolation of pancreatic islets, control incubations with this enzyme preparation were performed Incubation of glucagon with 0 2 μg of collagenase in place of homogenized islets for 60 min under identical conditions resulted in 0 2—0 4% increase of trichloroacetic acid — soluble radioactivity In the absence of glutathione, even collagenase concentrations up to 1 μg per incubation did not produce acid-soluble radioactivity exceeding 1% of the total acid-precipitable hormone-bound radioactivity

On the other hand, α-amylase activity was determined as a marker for exocrine tissue present in the islet preparations. Table I shows that contamination proved to be less than 5%

Discussion

Our results show that homogenate preparations of normal pancreatic islets contain both the thiol-protein disulfide oxidoreductase and the proteolytic enzyme system capable of degrading insulin in the sequential order. In islet cell tumors, however, the A and B chain-degrading proteinases are apparently absent [15], or in an inactive state The presence of residual crude collagenase used in the isolation of islets as a responsible factor for nonspecific proteolytic activities could be ruled out by the control incubations with collagenase and by extensive washings (8 times) of the islets with Hanks' solution After 5 washings, no collagenolytic activity could be detected in the suspension medium [16], as measured using the method described by Wunsch and Heidrich [13] Moreover, pancreatic islets prepared by microdissection only [17] displayed a proteolytic activity comparable with the collagenase-isolated ones Previous fluorimetric experiments which were performed to detect activity of typical exocrine enzymes, i e trypsin and carboxypeptidase B, showed that the proteolytic activity of the islets completely differed in its pH optimum and activation by SH-compounds from those of the exocrine tissue (Zuhlke, H and Steiner, D F , ref 18 and unpublished results)

On the other hand, the effects of glutathione and EDTA (Figs. 1 and 2) and the failure of Tos-PheCH$_2$ Cl to inhibit the proteolytic activity of islets rule out interference by enzymes from the exocrine pancreas

The effects of EDTA on the degradation of A chain and glucagon suggest that metal-dependent neutral proteinases of the cytosol or cytoplasmic organelles rather than lysosomal cathepsins appear to be involved in the hormone degradation under the experimental conditions employed

Acknowledgements

We are grateful to Dr S Ansorge (Physiologisch-Chemisches Institut der Martin-Luther-Universitat Halle) for valuable help during this work and Dr M

Ziegler (Zentralinstitut für Diabetes Karlsburg) for the gift of ^{125}I-labeled glucagon. The valuable technical assistance of Mrs. H. Kansy and E. Franke is gratefully acknowledged.

References

1 Renold, A E (1972) Diabetes 21, Suppl 2619—2631
2 Humbel, R E (1965) Proc Natl Acad Sci U S 53, 853—859
3 Zahradnik, J M , Meek, J C and Bolinger, R E (1969) Metabolism 18, 172—174
4 Varandani, P T , Shroyer, L A and Nafz, M A (1972) Proc Natl Acad Sci U S 69, 1681—1684
5 Varandani, P T (1973) Biochim Biophys Acta 295, 630—636
6 Kohnert, K D , Knospe, S , Ziegler, M and Zuhlke, H (1971) in IInd Int Symp Diabetes mellitus (Magyar, I and Behringer, A , eds), Vol 2, pp 405—408, Verlag Medizinische Akademie, Vienna
7 Ansorge, S and Kaiser, B (1969) Wiss Z Karl-Marx-Univ Leipzig, Math -Naturwiss R 18, 559—568
8 Jørgensen, K H and Larsen, U D (1972) Horm Metab Res 4, 223—224
9 Varandani, P T (1966) Biochim Biophys Acta 127, 246—249
10 Lacy, P E and Kostianovsky, M (1967) Diabetes 16, 35—39
11 Hahn, H J , Lippmann, H G and Schulz, D (1970) Acta Biol Med Germ 25, 421—429
12 Lowry, O H , Rosebrough, N J , Farr, A L and Randall, R J (1951) J Biol Chem 193, 265—275
13 Wunsch, E and Heidrich, H G (1963) Hoppe-Seyler's Z Physiol Chem 333, 149—151
14 Bernfeld, P (1955) in Methods of Enzymology (Colowick, S P and Kaplan, N O , eds) pp 149—158, Academic Press, New York
15 Varandani, P T (1974) Biochem Biophys Res Commun 60, 1119—1126
16 Hahn, H J , Jahr, H , Kohnert, K D and Zuhlke, H (1975) Horm Res , in the press
17 Hellerstrom, C (1964) Acta Endocr Copenh 45, 122—132
18 Zuhlke, H and Steiner, D F (1975) Fed Proc 34, 675 (Abstract)