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Drosophila Insulin Degrading Enzyme and Rat Skeletal Muscle Insulin Protease Cleave Insulin at Similar Sites†

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Received February 18, 1988; Revised Manuscript Received October 17, 1988

ABSTRACT: Insulin degradation is an integral part of the cellular action of insulin. Recent evidence suggests that the enzyme insulin protease is involved in the degradation of insulin in mammalian tissues. *Drosophila*, which has insulin-like hormones and insulin receptor homologues, also expresses an insulin degrading enzyme with properties that are very similar to those of mammalian insulin protease. In the present study, the insulin cleavage products generated by the *Drosophila* insulin degrading enzyme were identified and compared with the products generated by the mammalian insulin protease. Both purified enzymes were incubated with porcine insulin specifically labeled with ^{125}I on either the A19 or B26 position, and the degradation products were analyzed by HPLC before and after sulfitolysis. Isolation and sequencing of the cleavage products indicated that both enzymes cleave the A chain of intact insulin at identical sites between residues A13 and A14 and A14 and A15. Sequencing of the B chain fragments demonstrated that the *Drosophila* enzyme cleaves the B chain of insulin at four sites between residues B10 and B11, B14 and B15, B16 and B17, and B25 and B26. These cleavage sites correspond to four of the seven cleavage sites generated by the mammalian insulin protease. These results demonstrate that all the insulin cleavage sites generated by the *Drosopohila* insulin degrading enzyme are shared in common with the mammalian insulin protease. These data support the hypothesis that there is evolutionary conservation of the insulin degrading enzyme and further suggest that this enzyme plays an important role in cellular function.

Insulin is an important modulator of cellular growth and metabolism. The mechanisms by which insulin exerts its effects on cells are as yet incompletely understood, but certain components of the system have been characterized. The insulin signaling system includes the hormone, a specific receptor on the responding cell membrane, a mechanism for signal transmission that may involve intracellular messengers, and a degradative process for removing and inactivating the hormone.

Both the biological response and degradation of the hormone are initiated by binding of insulin to a specific membrane receptor (Terris & Steiner, 1975). The insulin receptor consists of two subunits, an α subunit which contains the primary binding site and a β subunit which has tyrosine kinase activity and appears to be important in propagating the intracellular signal (Jacobs et al., 1979; Massague et al., 1980; Kasuga et al., 1981; Roth & Cassel, 1983). Subsequent to binding, insulin is internalized via the endosomal pathway and ultimately degraded (Bergeron et al., 1985; Gordon et al., 1980). Some of the membrane-bound insulin is also degraded without requiring rapid internalization (Hamel et al., 1987).

Although the degradative pathway is not as well characterized, many lines of evidence now suggest that a metallo thiol

† This work was supported by Veterans Administration Research Funds, Indiana University Diabetes Research and Training Center (NIH-P60-AM-20542), National Cancer Institute Award CA35541 to M.R.R., and a grant from the Wade Research Fund to M.R.R.

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0006-2960/89/0428-2471$01.50/0

proteinase, variously termed insulin protease or insulin degrading enzyme, is the primary enzyme involved in initiating cellular insulin degradation in mammals. This conclusion has been supported by kinetic and inhibitor studies (Duckworth & Kitabchi, 1981), inhibition studies with antibodies against the protease (Shii & Roth, 1986), and the recent finding that the degradation products of insulin protease are identical with those of intact hepatocytes (Duckworth et al., 1988) and kidney (Duckworth et al., 1989). Thus, it appears that a specific insulin degrading enzyme is an important component of the mammalian insulin system.

Submammlian species also have insulin-like hormones and insulin binding homologues of the insulin receptor (LeRoith et al., 1986). An insulin-like hormone has been demonstrated in *Drosophila* and other insects including the blowfly, *Calliphora vomitoria*, *Hymerroptera*, *Orthoptera*, and the silkworm (Duve & Thorpe, 1979; Duve et al., 1979; Nagasawa et al., 1984; Meneses & Ortiz, 1975; LeRoith et al., 1981; Tager et al., 1976; Thorpe & Duve, 1984). An insulin receptor homologue from *Drosophila* that binds mammalian insulin with high affinity has also been characterized (Petruzelli et al., 1985a,b).

Recently, an enzyme has been isolated and purified from *Drosophila* that degrades insulin with considerable specificity. The *Drosophila* insulin degrading enzyme has a number of properties similar to those of mammalian insulin protease, including a molecular weight of 110000 and sensitivity to sulfhydryl inhibitors and to bacitracin (Garcia et al., 1988). This enzyme can be affinity labeled with insulin, as has been shown for the mammalian enzyme, and also binds epidermal growth factor (Thompson et al., 1985; Garcia et al., 1987, 1988). The studies described in the present paper were performed to examine and identify the degradation products of insulin generated by the *Drosophila* enzyme and compare them with the products produced by insulin protease from rat skeletal muscle. The result indicate that the primary sites of insulin degradation by both the mammalian and *Drosophila* enzymes are similar.

Materials and Methods

[^{125}I]Iodoinsulin (porcine) specifically labeled on either the A19 or the B26 tyrosine (Frank et al., 1983) was purified on a Sephadex G-25 column (0.7 × 25 cm) equilibrated with 0.1 M Tris-HCl, pH 7.5. One-milliliter fractions were collected and the peak tubes pooled. To 2 mL (approximately 2×10^7 cpm or 57 ng) of each isomer was added 50 μL of *Drosophila* insulin degrading enzyme (approximately 10 μg of enzyme protein). The enzyme was purified from *Drosophila* Kc cells by successive chromatography on DEAE-Sephadex, hydroxylapatite, and butylagarose as previously described (Garcia et al., 1988). The mixture was incubated for 2 h at 37 °C. The reaction was stopped by the addition of 150 μg of unlabeled insulin and 150 μL of concentrated acetic acid, and the tube was placed on ice.

An additional 2 mL of each isomer was incubated with 200 μL of a 1:15 dilution of insulin protease (approximately 60 μg of enzyme protein) (Duckworth et al., 1972) for 15 min at 37 °C and the reaction stopped as above.

Aliquots of the reaction mixtures were frozen in liquid N_2 for later analysis on HPLC (Hamel et al., 1986) while the remainder of the digests was lyophilized and then sulfitolyzed. The sulfitolyzed digests were frozen for subsequent HPLC analysis.

Sulfitolysis of Insulin Degradation Products. Lyophilized material was dissolved in 0.4 mL of $(NH_4)_2HPO_4$, pH 8.5. Then 50 μL of 1 mg/mL pork insulin, 50 μL of 20 mM Na_4EDTA, and 0.23 g of urea (7.5 M final) were added, and the samples were adjusted to pH 8.5 with NH_4OH. Twenty milligrams each of sodium tetrathionate and sodium sulfite was dissolved in the sample and allowed to react for 20 min at room temperature (Hamel et al., 1987). The products were then analyzed by HPLC using an elution program designed to separate the A-chain and B-chain *S*-sulfonate derivatives and their peptide fragments (see below).

HPLC of Insulin Degradation Products. Iodoinsulin and degradation products were separated on HPLC using a $(NH_4)_2HPO_4$, pH 4.0–acetonitrile system in a series of isocratic and linear gradient steps, similar to one reported previously (Hamel et al., 1986). The times and acetonitrile concentrations were as follows: (1) 5 min at 23.75%; (2) 5-min gradient to 26.25%; (3) 30 min at 26.25%; (4) 30-min gradient to 27.50%; (5) 15-min gradient to 40%; (6) 4 min at 40%. Flow was maintained at 1 mL/min thoughout the elution. Fractions (0.5 mL) were collected in bovine serum albumin coated tubes containing 10 μg of unlabeled insulin and counted directly in a Tracor Analytic γ counter to determine the elution profile of radioactivity. Known amounts of the sample were also counted to determine recoveries. Recoveries of the injected radioactivity ranged from 85 to 110% in the experiments shown.

HPLC and Sequence Analysis of Sulfitolyzed B Chain and Its Fragments. Sulfitolyzed A and B chains and the peptides generated by sulfitolysis of degradation products were separated on HPLC by an extension of a method previously reported (Hamel et al., 1986, 1987). The buffer was $(NH_4)_2HPO_4$ as above, but at pH 6.5. The isocratic and linear gradient steps in acetonitrile concentration were as follows: (1) 5 min at 15%; (2) 15-min gradient to 23.75%; (3) 20 min at 23.75%; (4) 35-min gradient to 40%; (5) 10 min at 40%. Fractions were collected as described above. Recoveries averaged 92% in the experiments shown. Peaks of radioactivity were pooled and lyophilized. The lyophilized material was desalted on a Sep-Pak (Waters and Associates; Milford, MA) eluted with 80% acetonitrile in water adjusted to pH 6.5. Sep-Pak recoveries averaged 96%. The eluant was lyophilized before sequencing on a Beckman Model 890C sequenator.

Results

Purified insulin degrading enzyme from *Drosophila* was incubated with A19-labeled [^{125}I]iodoinsulin ([^{125}I]iodo-(A19)insulin) and an aliquot of the reaction mixture analyzed on reversed-phase HLPC (Figure 1, panel A). In addition to intact A19 insulin eluting at 28 min, at least eight peaks of degradation products were observed. Similarly, [^{125}I]iodo-(A19)insulin was incubated with purified insulin protease prepared from rat skeletal muscle and an aliquot analyzed on HPLC using an identical program (Figure 1, panel B). Nine product peaks were observed. Some of these correspond to the peaks in panel A, but there are also several differences. The complexity results from multiple cleavage sites in both A and B chains.

For identification of the A-chain cleavages, aliquots from the [^{125}I]iodo(A19)insulin reaction mixtures were sulfitolyzed to separate the A and B chains. The S-sulfonated derivatives of chains and fragments were analyzed on HPLC. The results with the *Drosophila* enzyme are shown in Figure 2, panel A, and those with insulin protease in Figure 2, panel B. In addition to intact sulfonated (A19) A chain which elutes at 42 min, two apparently identical peaks seen both in panel A and in panel B elute from the column at 11 and 25 min and are termed peak 1 and peak 2. The elution times of these peaks are identical for both enzymes. For the *Drosophila* enzyme,

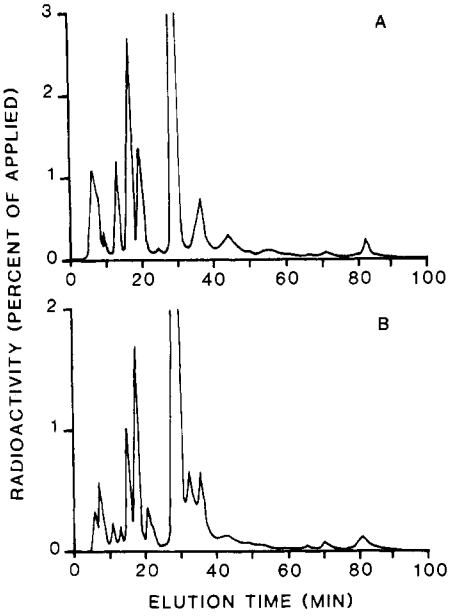

FIGURE 1: HPLC elution profile of [^{125}I]iodo(A19)insulin degraded by *Drosophila* insulin degrading enzyme and rat insulin protease. [^{125}I]Iodo(A19)insulin was incubated with *Drosophila* insulin degrading enzyme or with rat insulin protease. The reaction mixture was injected on reversed-phase HPLC, and the elution profile of radioactivity for the *Drosophila* enzyme is shown in panel A and that for insulin protease in panel B.

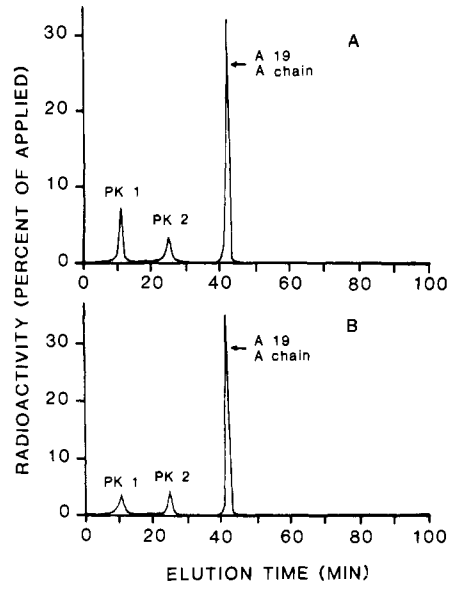

FIGURE 2: HPLC elution profile of sulfitolyzed products of *Drosophila* insulin degrading enzyme and rat insulin protease digestion of [^{125}I]iodo(A19)insulin. The reaction mixtures from the experiment shown in Figure 1 were sulfitolyzed and injected on reversed-phase HPLC. Panel A shows the profile of the *Drosophila* enzyme digestion and panel B the profile of insulin protease.

analysis of the reaction products indicates that peak 1 comprises 18.28% of recovered counts and peak 2 comprises 10.45%, with intact chain accounting for the remainder. For insulin protease enzyme products, peak 1 is 9.12% and peak 2 is 12.43%. When more extensive degradation studies were done to the point where less than 5% of the starting substrate was undegraded, no additional peaks were observed for either enzyme. Thus, although the elution pattern of unsulfitolyzed products is different, both enzymes produce identical A-chain fragments suggesting that the different patterns shown in Figure 1 are due to differences in B-chain cleavage sites.

To test this possibility, each peak from the original, unsulfitolyzed HPLC profile (Figure 1) was collected, pooled, lyophilized, and sulfitolyzed. The sulfitolyzed peptides were then reinjected with the peptide separation program to determine the peptide composition of each peak. Table IA shows the peptide composition of the A19 insulin digest by the *Drosophila* enzyme. The first eluting peak, which is asymmetrical and obviously heterogeneous, was treated as two components with the earliest, larger segment at 6.5 min containing solely the peptide found in peak 1 and the shoulder at 7.5 min containing a mixture of the peptides found in peaks 1 and 2. The remainder of the peaks was primarily composed of only one of these two A-chain peptides, although the broad peak at 43–45 min contained both. Since unsulfitolyzed peaks eluting at such widely different times (e.g., the peak at 7 and at 43 min) contained the same A-chain fragment, obviously different portions of the B chain were attached to the A-chain fragment.

Table I: Peptide Composition of HPLC-Purified Product Peaks after Incubation of [^{125}I]Iodo(A19)insulin with *Drosophila* Insulin Degrading Enzyme and with Rat Insulin Protease

elution time of unsulfitolyzed peaks (min)	peptide composition after sulfitolysis[a]
	(A) *Drosophila* Insulin Degrading Enzyme
6	peptide 1
7	peptide 1 (70%) + peptide 2 (30%)
9	peptide 2
13	peptide 1
17	peptide 1
19	peptide 2
35	peptide 1 + intact A chain
43	peptide 2 (70%) + peptide 1 (30%)
82	intact A chain
	(B) Rat Insulin Protease
6	peptide 1
8	peptide 2
11	peptide 2
13	peptide 1
15	peptide 1
18	peptide 2
21	peptide 2
33	peptide 2 (80%) + intact A chain (20%)
36	peptide 1 (15%) + peptide 2 (20%) + intact (60%)

[a] Peptides 1 and 2 refer to peaks 1 and 2, respectively, in Figure 4.

Table IB shows the peptide composition of A19 insulin products generated by the rat insulin protease. Again, most of the peaks contain a single A-chain fragment, although the two peaks eluting immediately after intact insulin are heterogeneous.

To identify the two peptide fragments generated by each enzyme and to determine the cleavage sites responsible for their production, the sulfitolyzed peptides were collected from the HPLC runs, desalted on Sep-Paks, and sequenced by automated Edman degradation. The cycle in which radioactivity appears identifies the position of the A19 tyrosine relative to the amino terminus. The results of the sequencing are shown in Figure 3. The iodinated tyrosine from the peptide in peak 1 elutes in the fifth cycle, indicating a cleavage occurred between A14 and A15, and the iodinated tyrosine from the peptide in peak 2 elutes in the sixth cycle, indicating a cleavage occurred between A13 and A14. These results confirm our previuos findings (Hamel et al., 1986; Duckworth et al., 1987) as well as reports from other laboratories (Muir et al., 1986; Davies et al., 1986) on the cleavage sites in the A chain produced by insulin protease. The identities of the two A-chain peptides also indicate that the differences in elution of unsulfitolyzed products (Figure 1) are due to differences in B-chain cleavages.

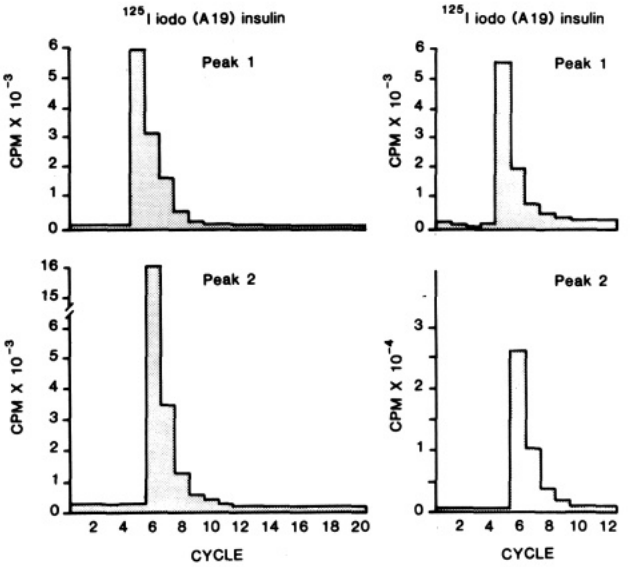

FIGURE 3: Quantitative sequence data for labeled peptides derived from degradation of [^{125}I]iodo(A19)insulin by *Drosophila* insulin degrading enzyme and insulin protease. The peptides shown in Figure 2, panel A, were submitted to automated Edman degradation, and each cycle was counted in an autogamma spectrophotometer.

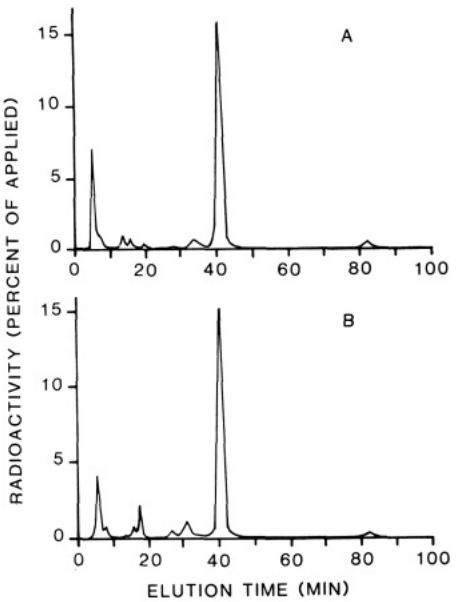

FIGURE 4: HPLC elution profile of [^{125}I]iodo(B26)insulin degraded by *Drosophila* insulin degrading enzyme and rat insulin protease. Conditions were as in Figure 1 except for the use of [^{125}I]iodo-(B26)insulin. *Drosophila* enzyme results are in panel A and those of insulin protease in panel B.

Similar studies were therefore done with [^{125}I]iodo(B26)-insulin incubated with each of the enzymes. The elution pattern for the *Drosophila* insulin degrading enzyme is shown in Figure 4, panel A, and that for the rat muscle insulin protease in Figure 4, panel B. Again, several products can be identified in addition to intact B26 which elutes at 61 min. Similar profiles were obtained under conditions where over 95% of the original substrate was degraded (data not shown). The elution pattern of sulfitolyzed reaction mixtures is shown in Figure 5. With the *Drosophila* protease (panel A), four product peaks can be identified in addition to intact B26 B chain. These elute at 5, 28, 41, and 63 min. The peak eluting at 63 min, labeled peak 4, does not separate well from the intact B-chain peak in this elution program. Panel B shows the elution profile of sulfitolyzed B-chain peptides produced by insulin protease. Seven peaks of products corresponding

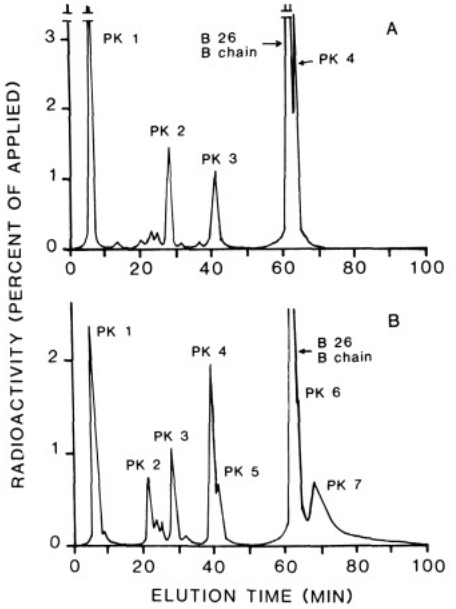

FIGURE 5: HPLC elution profile of sulfitolyzed products of *Drosophila* insulin degrading enzyme and rat insulin protease digestion of [^{125}I]iodo(B26)insulin. Conditions were as described in Figure 2 except for the use of [^{125}I]iodo(B26)insulin. *Drosophila* enzyme results are in panel A and those of insulin protease in panel B.

Table II: Percentage Composition of Sulfitolyzed B-Chain Fragments of [^{125}I]Iodo(B26)insulin Digestion by *Drosophila* Insulin Degrading Enzyme and Insulin Protease

peak from insulin protease digestion[a]	percent-age of recovered counts	peak from *Drosophila* enzyme[b]	percent-age of recovered counts
1	5.76	1	14.88
2	2.10		
3	2.43	2	4.56
4	6.27		
5	1.30	3	4.23
6	2.12	4	7.24
7	8.26		
intact B chain	64.79	intact B chain	61.51

[a] Peak numbers from Figure 5, panel B. [b] Peak numbers from Figure 5, panel A.

to seven fragments containing the B26 residue can be seen. These are numbered 1–7. Peak 5 at 41 min and peak 6 at 63 min do not separate well from peak 4 and intact B chain, respectively, in this elution program but do separate on other systems (Duckworth et al., 1988, 1989). This pattern is identical with our previous studies characterizing the products of insulin cleavage in vivo (Duckworth et al., 1988, 1989).

The retention times of the four B26 insulin degradation products generated by the *Drosophila* enzyme are identical with those four of the seven peaks generated by the rat insulin protease. Peaks 1–4 from the *Drosophila* enzyme digest (Figure 4, panel A) correspond to peaks 1, 2, 5, and the incompletely resolved peak 6, respectively, in the rat enzyme digest (Figure 4, panel B). No fragments from the *Drosophila* enzyme digest elute with the retention times of peaks 4 and 7 from the rate enzyme digest, but a small peak corresponding to peak 2 in the rat enzyme digest can be detected. The percentage of peptide in each peak of the digest is shown in Table II. The four peaks from the *Drosophila* enzyme digest were collected, desalted, and rechromatographed to obtain homogeneous peptide. These peptides were sequenced to determined the B-chain cleavage sites (Figure 6). We have previously sequenced all of the rat insulin protease fragments

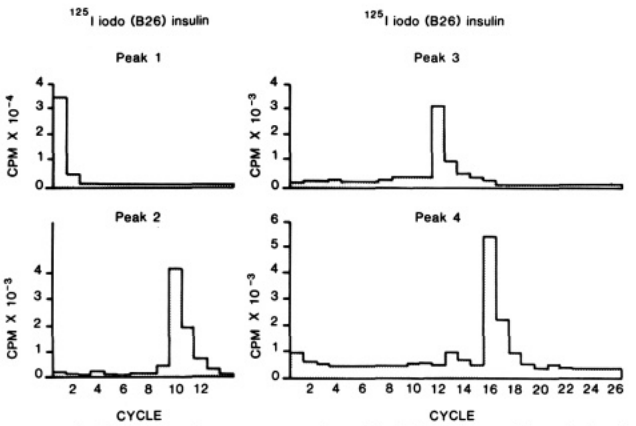

FIGURE 6: Quantitative sequence data for labeled peptides derived from degradation of [^{125}I]iodo(B26)insulin by *Drosophila* insulin degrading enzyme.

Table III: Peptide Composition of HPLC-Purified Product Peaks after Incubation of [^{125}I]Iodo(B26)insulin with *Drosophila* Insulin Degrading Enzyme and with Rat Insulin Protease

elution time of unsulfitolyzed peaks (min)	peptide composition after sulfitolysis[a]
	(A) *Drosophila* Insulin Degrading Enzyme
6	peptide 1
7	peptide 2
14	peptide 3
16	peptide 3
20	peptide 2
29	peptide 3
33	peptide 4
82	peptide 4 + intact B chain
	(B) Rat Insulin Protease
6	peptide 1 (75%) + peptide 2 (25%)
8	peptide 3
14	peptide 5
16	peptide 4 (50%) + peptide 5 (50%)
18	peptide 4
27	peptide 7
31	peptide 7 + peptide 6
82	intact peptide 6

[a] Peptides 1–7 refer to peaks 1–7, respectively, in Figure 5.

(Duckworth et al., 1988, 1989), but peaks 1, 2, 3, 4, and 7 (Figure 4, panel B) were resequenced and confirmed our previous results (data not shown). The *Drosophila* enzyme peak 1, like the rat protease peak 1, resulted from cleavage at B25-B26 and represented the major degradation product for both enzymes. The iodinated tyrosines from the *Drosophila* enzyme peak 2 (Figure 5, panel A) and the rat enzyme peak 3 (Figure 5, panel B) both eluted in the 10th cycle during sequencing, indicating a cleavage occurred between B16 and B17. Peak 3 from the *Drosophila* enzyme digest was generated by cleavage at B14-B15 and corresponded to peak 5 from the rat enzyme digest. Radioiodinated tyrosine from peak 4 of the *Drosophila* enzyme digest eluted in the 16th cycle, indicative of a B10-B11 cleavage, as shown previously for peak 6 from the rat insulin protease digest. Peaks 4 and 7 from the rat enzyme degradation products were generated by cleavages at B13-B14 and B9-B10, respectively. Thus, the four B-chain cleavages produced by the *Drosophila* insulin degrading enzyme correspond to four of the seven cleavages produced by the rat insulin protease.

Each of the original unsulfitolyzed peaks was also collected, sulfitolyzed, and reinjected on HPLC to determine peptide composition. The results are shown in Table III. The peak eluting at 6 min from the *Drosophila* enzyme digestion is

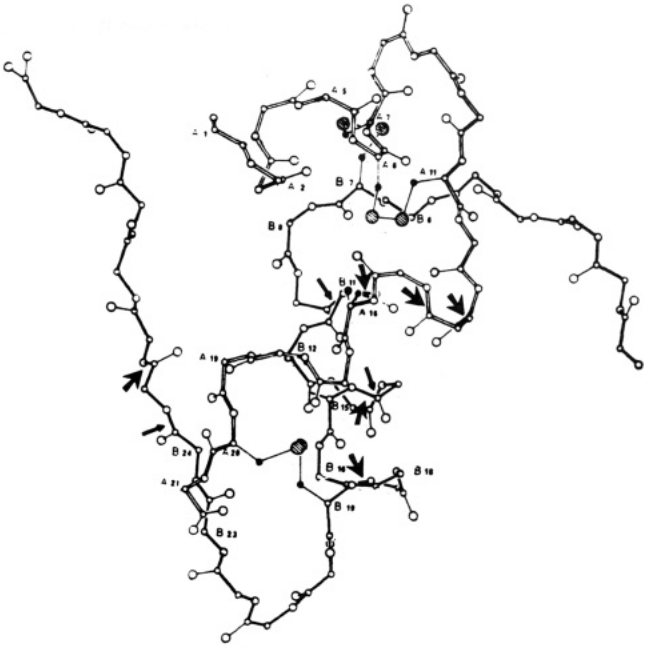

FIGURE 7: Schematic diagram of insulin showing sites of degradation shared by the *Drosophila* insulin degrading enzyme and the rat insulin protease (large arrows) and the additional cleavages of the rat enzyme (small arrows).

composed of the peptide cleaved at B25-B26, while the peak eluting at 6 min from the insulin protease digestion contains that peptide in addition to the one cleaved at B24-B25. The 8-min peak from both enzyme digestions contains the fragment resulting from the cleavage at B16-B17. The 14-min peak from both enzyme digestions contains the products from cleavage of the B13-B14 bond; the 16-min peak from the rat enzyme degradation contains peptides derived from cleavage at B14-B15 as well as at B13-B14. The rat protease-derived peak that elutes at 18 min results from a B14-B15 cleavage. The 33-min *Drosophila* enzyme peak is derived from a B10-B11 cleavage, while the 27- and 31-min rat protease peaks contain this peptide plus a peptide cleaved at B11-B12. The late eluting peak (41 min) reflects a B10-B11 cleavage for the *Drosophila* enzyme and a B11-B12 cleavage for insulin protease.

Discussion

In the present study we have demonstrated that the products of insulin degradation by the *Drosophila* and the mammalian enzymes are very similar. Intact insulin degradation by rat insulin protease results in two cleavages in the A chain and seven in the B chain (Duckworth et al., 1988, 1989, 1987). The seven B-chain cleavages occur in four regions of the polypeptide, one comprising the B9-B10 and B10-B11 sites, a second appearing in the vicinity of B13-B14 and B14-B15, a third consisting of B16-B17, and a fourth containing the more distant B24-B25 and B25-B26 sites. The *Drosophila* enzyme cleaves the A chain of insulin at the same two cleavage sites as the rat insulin protease and also shares one of the two cleavage sites in each of the four regions of the B chain (summarized in Figure 7). For both the *Drosophila* and rat enzymes, the primary end product of insulin degradation results from cleavage at residues 25–26 of the B chain [see also Duckworth et al. (1988, 1989)].

All of the additional rat insulin protease degradation sites are amino terminal to the primary cleavage sites for the *Drosophila* enzyme. This result raises the possibility that these additional cleavages might derive from the action of a con-

taminating carboxypeptidase in the insulin protease preparation. However, if that were the case, no peptides with the primary sites uncleaved (e.g., peptides 2, 4, and 7, Figure 5B) would have been observed. Another possibility is that the mammalian preparation contains two endoproteinases that generate additional cleavage sites. This scenario is also unlikely since all proteolytic activity in the preparation can be removed by immunoprecipitation with a monoclonal antibody to the erythrocyte insulin degrading enzyme (Shii et al., 1986) and the immunoprecipitated proteases from both muscle and red blood cells produce the same cleavages (unpublished observations).

Mammalian insulin is not a physiological substrate for the *Drosophila* enzyme. However, insulin has been shown to support the growth of *Drosophila* cells, suggesting that the mammalian hormone can mimic a physiological interaction within the *Drosophila* organism. It will certainly be of interest to examine the effects of both the *Drosophila* and rat enzymes on the *Drosophila* insulin-like peptide when this material becomes available for study. Although neither the *Drosophila* nor rat enzymes degrade procine insulin in vivo, our results demonstrate that the enzymes from both of these evolutionarily distant species retain similar specificities for a heterologous substrate.

The multiple bonds cleaved by insulin protease preclude a simple classification of peptide bond specificity for the proteinase. However, examination of the three-dimensional structure of insulin shows that all of the bonds cleaved by the enzyme except B24-B25 and B25-B26 are in close proximity. This observation suggests that the specificity of the enzymes is directed to the molecule itselt rather than to particular peptide bonds [see also Duckworth et al. (1988, 1989)]. The reasons for such an unusual specificity are at present unclear. An extensive literature review revealed no reported proteinase from mammalian or bacterial origin with comparable specificities (Duckworth et al., 1988, 1989). Therefore, although we cannot dismiss the possibility that an unrelated protease could have identical cleavage sites, the high degree of conservation of the bonds cleaved strongly supports previous evidence (Garcia et al., 1988) for a direct evolutionary relationship between *Drosophila* and mammalian insulin degrading enzymes.

Although numerous lines of evidence support the potential importance of the insulin protease in the physiological degradation of insulin, the actual mechanism of cellular insulin degradation remains controversial. Degradation of insulin ensues at a very early step during receptor-mediated endocytosis, since degradation products can be found in early endosomes (Hamel et al., 1988). In *Drosophila*, the insulin degrading enzyme is developmentally regulated, being barely detectable in the embryo but elevated 5-fold in the larvae and pupae and 10-fold in the adult. These findings suggest that the enzyme plays a specific role in the later stages of the life cycle of the fly (Stopelli et al., 1988) and complement evidence in mammalian systems that degradation may influence at least some of the actions of insulin (Peavy et al., 1984). In sum, the present data and the previous findings suggest that the insulin degrading enzyme is evolutionarily conserved and may play a critical role in cellular function.

ACKNOWLEDGMENTS

We thank Brad Fenton for his assistance and M. Patrizia Stoppelli for helpful discussions.

Registry No. Insulin, 9004-10-8; insulinase, 9013-83-6.

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Distribution of Lipid-Binding Regions in Human Apolipoprotein B-100†

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Received August 8, 1988; Revised Manuscript Received October 9, 1988

ABSTRACT: The distribution of lipid-binding regions of human apolipoprotein B-100 has been investigated by recombining proteolytic fragments of B-100 with lipids and characterizing the lipid-bound fragments by peptide mapping, amino acid sequencing, and immunoblotting. Fragments of B-100 were generated by digestion of low-density lipoproteins (LDL) in the presence of sodium decyl sulfate with either *Staphylococcus aureus* V8 protease, pancreatic elastase, or chymotrypsin. Particles with electron microscopic appearance of native lipoproteins formed spontaneously when detergent was removed by dialysis from enzyme digests containing fragments of B-100 and endogenous lipids, or from incubation mixtures of delipidated B-100 fragments mixed with microemulsions of exogenous lipids (cholesteryl oleate and egg phosphatidylcholine). Fractionation of the recombinant particles by isopycnic or density gradient ultracentrifugation yielded complexes similar to native LDL with respect to shape, diameter, electrophoretic mobility, and surface and core compositions. Circular dichroic spectra of these particles showed helicity similar to LDL but a somewhat decreased content of β-structure. Most of the fragments of B-100 were capable of binding to lipids; 12 were identified by direct sequence analysis and 14 by reaction with antisera against specific sequences within B-100. Our results indicate that lipid-binding regions of B-100 are widely distributed within the protein molecule and that proteolytic fragments derived from B-100 can reassociate in vitro with lipids to form LDL-like particles.

Low-density lipoproteins (LDL),[1] the major carriers of cholesterol and cholesteryl esters in human plasma, are spherical particles (18–25 nm in diameter) that consist of a neutral lipid core (mainly composed of cholesteryl esters) surrounded by a polar surface shell (phospholipids, unesterified cholesterol, and protein) (Deckelbaum et al., 1977; Gotto et al., 1986). Apolipoprotein B-100 is the sole protein component of LDL and is the ligand responsible for the binding of LDL to the LDL receptor (Brown & Goldstein, 1986; Mahley et al., 1977, 1984). Moreover, B-100 is one of the largest single polypeptides known, consisting of 4536 amino acid residues, as deduced from cDNA clones (Knott et al., 1986; Law et al., 1986; Yang et al., 1986), and appears to possess a very high affinity for lipids, exemplified by its inability to transfer among lipoprotein particles and by its insolubility in aqueous media after delipidation. This high affinity for lipids suggests a structural role for B-100 in the formation and maintenance of lipoprotein particles (Kane, 1983).

Various model LDL systems have been employed to study the detailed structural organization of LDL particles and the nature of the interaction of B-100 with lipids (Atkinson & Small, 1986). These systems involve the reassembly of intact B-100 with surface phospholipids alone (Watt & Reynolds, 1981; Walsh & Atkinson, 1983; Dhawan & Reynolds, 1983) or with surface and core lipids (Krieger et al., 1978; Ginsburg et al., 1984; Lundberg & Suominen, 1984). Such systems have clearly demonstrated the ability of B-100 to bind lipids and to form model LDL complexes that exhibit some physicochemical and biological properties similar to those of native LDL. In addition, small tryptic peptides (M_r <5000) (Cardin & Jackson, 1986) and large thrombolytic fragments (Corsini et al., 1987) of B-100 also possess high enough affinity for lipids to form stable complexes when interacting with dimyristoylphosphatidylcholine liposomes or cholesterol-induced canine high-density lipoproteins, respectively. However, few details are known about the distribution of lipid-binding regions

† This work was supported by National Institutes of Health Grants HL 14237 (Arteriosclerosis Specialized Center of Research), HL 01546 (Clinical Investigator Award), and AM 26743 and by a grant from the Muscular Dystrophy Association.

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[1] Abbreviations: LDL, low-density lipoprotein(s); VLDL, very low density lipoprotein(s); apo B, apolipoprotein B; SP, *Staphylococcus aureus* V8 protease; EL, pancreatic elastase; CH, chymotrypsin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); K_D, equilibrium dissociation constant; CD, circular dichroism.

0006-2960/89/0428-2477$01.50/0