Identification of Insulin Intermediates and Sites of Cleavage of Native Insulin by Insulin Protease from Human Fibroblasts*

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We have studied the time sequence degradation of native insulin by insulin protease from human fibroblast using multiple steps involving purification of the products by high performance liquid chromatography, determination of peak composition by amino acid sequence analysis, and confirmation of structure by mass spectrometry and thus elucidated the sites of cleavage of insulin by human insulin protease. We observed that as early as 0.5 min of incubation, three major new peptide bonds, intact insulin, and four smaller peptide peaks can be detected. The major peptide bonds are portions of the insulin molecule, with the amino ends of the A and B chains or the carboxyl ends of the A and B chains still connected by disulfide bonds. Peptide peak I is A1-11-B12-13. Peptide peak II is A1-13-B14-15. Peptide peak III is A1-14-B15-16. The smaller peptide peaks are A14-21, B17-30, A15-21, B14-30, A15-21-B10-30, and A14-21-B10-30. The major peptide bond cleavage sites therefore consist of A13-14, A14-15, B9-10, B13-14, and B16-17. With longer incubation times, peptide peak II appears to lose the A14 tyrosine to form peptide peak I. This peptide I, which is the amino end of the A and B chains, is not further degraded even after 1.5 h of incubation. With longer incubation times, the peptides containing the carboxyl ends of the A and B chains are further degraded to form products from cleavage at the A18-19, B14-15, B9-10, and a small amount of A14-20-B9-11, and B24-25 cleavage and the emergence of 2-5-amino acid peptide chains, tyrosine, alanine, histidine, and leucine-tyrosine. We conclude, based on the three-dimensional structure of insulin, that human insulin protease recognizes the α-helical regions around leucine-tyrosine bonds and that final degradation steps to small peptides do not require lysosomal involvement.

The mechanism by which insulin exerts its biological effects is still unclear. Studies have shown that initially, insulin binds to its specific receptor on the cell membrane of insulin-sensitive tissues (1, 2) and is then internalized through receptor-mediated endocytosis (3-7). Subsequent to binding, insulin activates the receptor tyrosine kinase activity (8-12), begins to exert its biological effects, and cellular processing of insulin occurs with the degradation of insulin into insulin intermediate molecules and eventually low molecular weight trichloroacetic acid-soluble peptides using A14,15-F-insulin, as shown by us and others, using both human cells and other animal tissue (13-23). Cell-mediated degradation of insulin has been demonstrated in all insulin-sensitive mammalian tissues (24, 25), with a few of these studies in human tissue. Although binding to the insulin receptor is an essential step for insulin degradation (25), the mechanism by which the enzyme(s) then degrade insulin once it is bound and then internalized (26) is unclear. Internalized insulin has been found in various fractionated components of the cell such as cytoplasm, Golgi, endoplasmic reticulum, nucleus, and lysosomes (27-29). Three enzyme systems implicated in the degradation of insulin are: (a) soluble neutral insulin protease (14, 30-32); (b) glutathione-insulin transhydrogenase, a microsomal reduction cleavage enzyme (33); and (c) lysosomal acid protease (34). Glutathione-insulin transhydrogenase and lysosomal degradation appear to be only minimally involved in insulin degradation since most of the insulin degradation occurs before it reaches the lysosomes, and the acidic proteases and glutathione-insulin transhydrogenase show minimal insulin-degrading activity (35) when compared with the neutral insulin protease isolated in the cellular cytosolic fraction (30, 32, 36-39). Studies, including inhibitors, kinetic data, and antibody, indicated insulin protease to be the primary cellular insulin-degrading enzyme (14, 24, 25, 30, 32, 39-41). How this cytosolic insulin protease can degrade in-

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insulin once it is bound to its receptor has been uncertain; however, recent studies have shown that this enzyme is capable of degrading receptor-bound insulin (42).

Although the relationship of insulin degradation to its mechanism of action is not known, it has been suggested that the enzyme may be important in either terminating (43) or generating signals (44) for insulin action. Additionally, it is possible that the insulin intermediates formed by insulin protease (13) may act as putative mediators for some of the actions of insulin such as stimulation of pyruvate dehydrogenase (45). As early as 1955, alterations in the metabolism of insulin had been implicated in contributing to the state of insulin resistance in animals (46), and more recently, studies have shown that the activity of insulin protease affects the sensitivity and resistance to insulin in certain clinical conditions (47, 48). In order to study the effects of these insulin intermediates on the various insulin-sensitive biological actions, sufficient insulin intermediates need to be made, isolated, and the structure of these intermediates determined. Using $^{14}$C-125I-insulin or other radioactively labeled insulin, it is virtually impossible to make and isolate sufficient intermediates, and even if it were possible, the amount of radioactivity needed would be very high for sequence and molecular mass determinations. There is also the problem of the effect

![Fig. 1. HPLC elution pattern of insulin intermediates generated using incubation of unlabeled insulin with human insulin protease for 0 min (A), 0.5 min (B), and 2 min (C). Each incubation mixture was injected onto a reverse phase C$_{18}$ 25-cm Vydac column and separated with a nine-step gradient system of acetonitrile, 0.1% trifluoroacetic acid/H$_2$O. The concentration of acetonitrile in each step is shown in A.](image-url)
that the radioactive iodine on the molecule might have on the biological systems and the problem that some of the intermediates might not have the radioactive label and therefore escape detection. Additionally, the number of intact cells required to isolate sufficient radioactive or cold intermediates would be prohibitive. Since our earlier studies (32) showed the same two major radioactive intermediate insulin peaks with intact fibroblast or insulin protease purified from the fibroblasts, we used the protease for these studies. We have developed a method to isolate the native insulin and intermediates and have determined the structure of these intermediate products from normal human fibroblasts. We have studied time sequence degradation of native insulin by the method we developed using multiple steps involving purification of the intermediates by HPLC,1 sequencing of each peak using an amino acid sequenator, and confirming the structure of these insulin intermediates by mass spectrometry. Determination of the structure of the insulin intermediates hence elucidates the sites of cleavage of insulin by insulin protease.

EXPERIMENTAL PROCEDURES

Materials—Crystalline porcine insulin was kindly provided by Dr. Robert Chance of Eli Lilly Company. Trifluoroacetic acid, Sequanal grade, was obtained from Pierce Chemical Co.; acetonitrile (ACN), distilled in glass, was obtained from Burdick and Jackson (Muskegon, MI). Sep-Pak C<sub>18</sub> cartridges were obtained from Millipore-Waters Division (Milford, MA). Vycod C<sub>18</sub> reverse phase protein and peptide column and guard column were obtained from the Nest Group (Southboro, MA). Ultrapure grade ammonium sulfate was obtained from Schwarz/Mann. Protein determinations were performed using Bio-Rad dye. Sephacyr G-200 HR, Sephadex G-50, QAE-Sephadex, and CNBr-activated Sepharose 4B were purchased from Pharmacia LKB Biotechnology Inc. A<sub>14</sub>-Insulin was prepared according to our method published previously (49). Fetal bovine serum and Eagle’s minimal medium were purchased from HyClone Laboratories (Logan, UT). Other tissue reagents were as described elsewhere (13).

Preparation of Insulin Protease—The human fibroblast cultures were established from human foreskins and maintained as described previously (13, 32, 50) using the method of Howard et al. (61). The cells used for the enzyme preparations were between the 8th and 12th passages. After reaching confluent monolayers and 2 days after the last addition of fresh Eagle’s minimal medium. The monolayers of human fibroblasts were washed four times with phosphate-buffered saline, pH 7.5, and then were scraped from the tissue culture flasks. The cells were then homogenized in 0.53 M sucrose and centrifuged at 100,000 × g at 4 °C. The supernatant was free of bacterial contamination as determined by enzymatic markers (32), was removed and fractionated with NH<sub>4</sub>SO<sub>4</sub>. The precipitate of the 30–60% ammonium sulfate fractionation was dissolved in 0.02 M sodium acetate and then dialyzed against 0.02 M sodium acetate. The enzyme preparation was then further purified on a Sephacryl G-260 HR column and then a QAE-Sephadex column and eluted with 0.2 M NaCl in 0.02 M sodium acetate, pH 6.2, and dialyzed against 0.02 M sodium acetate. The insulin-degrading activity in the fractions from each of the columns was determined by trichloroacetic acid precipitation of A<sub>14</sub>-insulin. Insulin protease was further purified for some of the degradation studies using insulin an affinity column made using CNBr-activated Sepharose 4B (14).

Insulin at a concentration of 1.67 × 10<sup>-5</sup> M in 0.01 M NaH<sub>2</sub>P<sub>0</sub><sub>4</sub>, pH 7.4, was incubated at 37 °C with 0.01 μg of human fibroblast insulin protease/10 ml of incubation volume for various times from 0 to 90 min. At the end of each incubation, the reaction was stopped with 50 μl of 0.1 M N-ethylmaleimide, pH 7.0, and the samples were injected onto a Perkin-Elmer series 400 HPLC with a Vydac C<sub>18</sub> column and guard column and LC-85B UV detector. The insulin and intermediates were separated using a nine-step gradient system of 0.1% trifluoroacetic acid/H<sub>2</sub>O, pH 3.0, and acetonitrile. The gradient system used was: step 1, 10% ACN, 90% 0.1% trifluoroacetic acid/H<sub>2</sub>O isocratic for 10 min; step 2, 10-min gradient to 20% ACN, 80% 0.1% trifluoroacetic acid/H<sub>2</sub>O; step 3, 10-min isocratic at 20% ACN, 80% 0.1% trifluoroacetic acid/H<sub>2</sub>O; step 4, 20-min gradient to 25% ACN, 75% 0.1% trifluoroacetic acid/H<sub>2</sub>O; step 5, 10-min isocratic at 25% ACN, 75% 0.1% trifluoroacetic acid/H<sub>2</sub>O; step 6, 10-min gradient to 28% ACN, 72% 0.1% trifluoroacetic acid/H<sub>2</sub>O; step 7, 20-min isocratic at 28% ACN, 72% 0.1% trifluoroacetic acid/H<sub>2</sub>O; step 8, 30-min isocratic at 80% ACN, 50% 0.1% trifluoroacetic acid/H<sub>2</sub>O, and the pump then mixed these two solutions in the proper proportions for the various gradient steps. The peaks were collected as they eluted and the solvent removed using the Speed-Vac concentrator. Seven preparations of each individual peak pooled from at least four HPLC separations were used for sequence analysis and mass spectrometry.

Reduction and alkylation of the cystine bonds in the insulin intermediates was performed using the procedure of Cleland (52). The products were dissolved in 1 ml of 0.01 M NaH<sub>2</sub>P<sub>0</sub><sub>4</sub>, buffer, pH 8.5, with 7 M urea. 10 μl of 1 mg/ml Cleland’s reagent (dithiothreitol) was added to the products, and then 10 μl of 1 mg/ml bromoacetic acid was added and the reaction was incubated for 10 min. The reaction mixture was then loaded onto a C<sub>18</sub> Sep-Pak and eluted with 80% ACN, 20% 0.1% trifluoroacetic acid/H<sub>2</sub>O and injected on the HPLC using the same gradient system as used for the initial product separation.

Gas-phase amino acid sequence analysis of 100–500 ng of each of the insulin intermediates (obtained from multiple pools of incubation mixtures) was performed through 30 cycles using an Applied Biosystems model 470 or 477 Microsequencer with standards and reagents according to manufacturer’s procedures. Fast atom bombardment mass spectrometry was performed using a VGZFE-2AB mass spectrometer from VG Instruments, Manchester, Great Britain. The insulin intermediates of approximately 1 pg/ml were analyzed using glycerol as solvent.

<table>
<thead>
<tr>
<th>Peptide peak number</th>
<th>Retention time (min)</th>
<th>Molecular mass (daltons)</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>41.5</td>
<td>A&lt;sub&gt;14&lt;/sub&gt;-Insulin</td>
<td>A&lt;sub&gt;14&lt;/sub&gt;-Insulin</td>
</tr>
<tr>
<td>II</td>
<td>46.0</td>
<td>A&lt;sub&gt;14&lt;/sub&gt;-Insulin</td>
<td>A&lt;sub&gt;14&lt;/sub&gt;-Insulin</td>
</tr>
<tr>
<td>III</td>
<td>54.2</td>
<td>A&lt;sub&gt;14&lt;/sub&gt;-Insulin</td>
<td>A&lt;sub&gt;14&lt;/sub&gt;-Insulin</td>
</tr>
<tr>
<td>IV</td>
<td>81.1</td>
<td>A&lt;sub&gt;14&lt;/sub&gt;-Insulin</td>
<td>A&lt;sub&gt;14&lt;/sub&gt;-Insulin</td>
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<tr>
<td>V</td>
<td>39.4</td>
<td>A&lt;sub&gt;14&lt;/sub&gt;-Insulin</td>
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<tr>
<td>VI</td>
<td>51.3</td>
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<tr>
<td>VII</td>
<td>68.4</td>
<td>A&lt;sub&gt;14&lt;/sub&gt;-Insulin</td>
<td>A&lt;sub&gt;14&lt;/sub&gt;-Insulin</td>
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<tr>
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<td>52.2</td>
<td>A&lt;sub&gt;14&lt;/sub&gt;-Insulin</td>
<td>A&lt;sub&gt;14&lt;/sub&gt;-Insulin</td>
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</table>
Insulin Intermediates Generated by Human Insulin Protease

RESULTS

Fig. 1A shows the HPLC elution profile, measured by UV absorbance at 210 nm, of the 0-min incubation of native insulin with the purified insulin protease. The percent ACN of the gradient systems used is also indicated. As can be seen, insulin (peak IV) at a retention time (RT) of 81 min is the only peak seen other than the area of the void volume (V₀) (RT = 3.2 min) and a baseline rise after 100 min, which is a column wash-off of some UV-absorbing materials on the C₁₈ column. The same peaks at the void volume and after 100 min were also observed in controls that were treated the same as the zero time and other time incubations except no insulin was added (data not shown).

Fig. 1B shows the HPLC elution profile after incubating insulin with the insulin protease for 0.5 min. Three major intermediate peptide peaks (I, II, and III) with RT of 41.5, 46.0, and 54.2 min, respectively, were then observed in addition to the insulin peak, which had decreased in peak height. Four other small peptide peaks were observed (V, VI, VII, and VIII) with RT of 39.4, 51.3, 68.4, and 71.8 min, respectively. Two small peptide peaks were also observed (1 and 2) which eluted just after the void volume at RT of 4.9 and 7.6 min, respectively. Longer incubations of 2 min (Fig. 1C) showed an increase in peak heights of I, II, and III, with a continued decrease in the peak height of insulin. One additional peptide peak (IX) was also observed at RT of 52.2 min.

Each of these peaks was collected and sequenced on an
TABLE II
Structure of later insulin degradation peaks generated by human insulin protease

<table>
<thead>
<tr>
<th>Peptide peak no.</th>
<th>Retention time</th>
<th>Molecular mass</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>daltons</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4.9</td>
<td>181, 155, 89, 294</td>
<td>Tyr, His, Ala, Leu-Tyr</td>
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<tr>
<td>2</td>
<td>7.6</td>
<td>496</td>
<td>B16-20 (His16-Glu20)</td>
</tr>
<tr>
<td>3</td>
<td>9.1</td>
<td>502</td>
<td>A15-18 (Gln15-Ala18)</td>
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<tr>
<td>4</td>
<td>20.1</td>
<td>519</td>
<td>B26-30 (Tyr26-Ala30)</td>
</tr>
<tr>
<td>5</td>
<td>23.4</td>
<td>1113</td>
<td>A20-33 (Cys20-Asn33)</td>
</tr>
<tr>
<td>6</td>
<td>28.1</td>
<td>1424</td>
<td>B17-29 (Leu17-Phe29)</td>
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<tr>
<td>7</td>
<td>33.0</td>
<td>2071</td>
<td>A14-21 (Tyr14-Asn21)</td>
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<tr>
<td>8</td>
<td>34.0</td>
<td>1985</td>
<td>B17-29 (Leu17-Phe29)</td>
</tr>
<tr>
<td>9</td>
<td>36.6</td>
<td>2469</td>
<td>A15-21 (Gln15-Asn21)</td>
</tr>
<tr>
<td>10</td>
<td>38.4</td>
<td>2494</td>
<td>B17-30 (Leu17-Ala30)</td>
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<tr>
<td>11</td>
<td>44.4</td>
<td>2657</td>
<td>A13-19 (Gly1-Ala19)</td>
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<td>48.3</td>
<td>2746</td>
<td>A5-21 (Gln5-Asn21)</td>
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<td>13</td>
<td>63.6</td>
<td>5216</td>
<td>B15-30 (Leu15-Ala30)</td>
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<td>14</td>
<td>70.6</td>
<td>3158</td>
<td>A25-31 (Gly25-Asn31)</td>
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<tr>
<td>15</td>
<td>73.6</td>
<td>3321</td>
<td>B17-30 (Leu17-Ala30)</td>
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<tr>
<td>16</td>
<td>75.8</td>
<td>5796</td>
<td>A14-21 (Tyr14-Asn21)</td>
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<td>17</td>
<td>76.1</td>
<td>5796</td>
<td>B1-14 (Phe1-Glu15, Ala16-Ala20)</td>
</tr>
<tr>
<td>18</td>
<td>77.7</td>
<td>5796</td>
<td>B18-30 (Phe1-Ser1, His16-Ala20)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A25-31 (Gly25-Asn31)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>B14-17 (Phe1-Tyr16, Leu17-Ala20)</td>
</tr>
</tbody>
</table>

amino acid sequenator, checked by amino acid composition, and also confirmed using mass spectrometry to determine the molecular mass of these peptides. Table I lists the peptide peaks, their retention times, structure, and molecular mass of the peptide peaks observed in Fig. 1, B and C. The peptides are portions of the amino end of the A and B chain still connected by the disulfide bond or portions of the carboxyl end of the A and B chains still connected by the disulfide bond. Peptide I is the amino end of the insulin A and B chains after insulin protease has cleaved the bond at the A18-20 (Leu18-Tyr20), and at the B20-21 (Ser20-His21) bond. Peptide III is the carboxyl end of the A chain consisting of A14-22 connected to the carboxyl end of the B chain consisting of B14-20. Peptide III is essentially the other half of the insulin molecule corresponding to peak I, with the 4-amino acid peptide B10-13 missing. Therefore, the B13-14 bond has been cleaved as well as the A13-14 bond to form peptide III. Peptide II, like peptide I, is the amino end of the A and B chains; however, in peptide II, the A14-15 (Tyr14-Gln15) bond has been cleaved. Peptide VI is the corresponding carboxyl end portion, similar to peptide III. Another B chain cleavage at B16-17 (Tyr16-Leu17) along with A18-24 forms peptide peak V. Peptides VII and VIII are the carboxyl ends generated with the formation of peptides II and I, respectively, without also cleaving the B13-14 bond. Peptide peak IX, which appears at 2 min of incubation, is the same as peptide III, but the B Ala14 has been clipped off. This peptide peak is probably generated from peptide III. Sulfotolysis of these major peaks and recromatographing them on the same HPLC gradient system showed no intact A or B chain of insulin which had retention times of 35.9 or 92.0 min, respectively.

Fig. 2, A-C, shows the HPLC of later incubation times of 5, 30, and 90 min, respectively. At 5 min of incubation, peptide peaks I and II have increased in height. However, by 30 min, peak II has begun to decrease and at 90 min has essentially all disappeared. The A14 tyrosine on the insulin intermediate (peak II) evidently is cleaved off at the A13-14 bond to form the intermediate A1-13-B1'-9 (peak I), which would explain the tyrosine in peak I. Peak 1 has continued to increase in size and is still large at 90 min. It is the only peak of the original major peaks still observed and does not appear to be further degraded by insulin protease.

Peptide peak III has reached a plateau at 5 min and then begins to decrease and by 50 min has totally disappeared, whereas peaks V and IX increased and then totally disappeared by 90 min, as well, in a pattern that indicates that the B24-25 is being cleaved on the insulin intermediates of peptide peaks III and IX. The other carboxyl end peptides VII, VIII, and IX began to decrease after 5 min and had totally disappeared by 90 min. The disappearance of these initial peaks of the carboxyl ends of insulin resulted in the appearance of the smaller, further degraded carboxyl end insulin pieces, peptide peaks 2-9 and 12, which we found to be formed from cleavage at bonds A19-20, A19-20, B25-26, and some B24-25. Additionally, at 5 min of incubation with insulin protease, several other small peaks appeared (Table II). Peaks 10, 11, 14, 15, and 15 are like peptides peaks I, II, VII, and VIII, respectively, except the B10-13 (His10-Leu13) bond is cleaved instead of the B11-12 (Ser11-His12) bond. It is possible that B10-13 (His10) is removed to yield peptide peak I since some free histidine was detected in peak I. Peptides 13, 16, 17, and 18 are insulin with B20-26, B20-14, B20-10, or B16-17 cleaved but still an intact A chain. These peaks are formed in very small amounts and are further degraded and undetectable at 90 min. These intermediates may represent an incomplete or misalignment in the insulin protease binding to the insulin molecule for degradation. Therefore, human insulin protease may clip one site on the B chain and not the A chain, but the major insulin products have been cleaved at both the A and B chains.

Since our studies with A14,12II-insulin (13) as well as those of Duckworth and others have shown that the major insulin radioactive intermediates elute with the insulin peak, and these intermediates that we have isolated would not seem to be large enough to elute with insulin, the 0-min and 5-min incubations were chromatographed on a molecular sieve column. It was surprising to find that peptide peaks I, II, and III, with molecular masses of approximately 2400-3500 daltons, would elute with the insulin peak on Sephadex G-50. Either the Stokes radius of these molecules is such that they do not elute according to actual molecular mass, or these essential halves of the insulin molecule are binding together through some noncovalent bonding to co-elute with the intact insulin molecule. Similar findings were reported by Asoian et al. (16, 17).

Fig. 3 depicts the initial peptide bonds cleaved by human
Insulin Intermediates Generated by Human Insulin Protease

**DISCUSSION**

This is the first study using human or other animal insulin protease in which the products of native insulin degradation (i.e., insulin intermediates) have been isolated and the actual amino acid sequence of the portions of the A and B chain determined as well as confirmed by mass spectrometry. We have observed that as early as 0.5 min, three major new peptide peaks, intact insulin, and four smaller peptide peaks can be detected which elute off a Sephadex G-50 column as peptide peaks, intact insulin, and four smaller peptide peaks.

**FIG. 3.** Peptide bonds cleaved and structure of insulin intermediates formed in 0.5 min of incubation of insulin with human insulin protease. This shows the structure of the intermediates observed in Fig. 1B.

**FIG. 4.** Rope model of the three-dimensional structure of insulin modified from Hodgkins (56). The black rope represents the A chain, and the clear rope is the B chain of insulin. The heavy arrows indicate the initial peptide bonds cleaved by human insulin protease. The smaller arrows indicate the bonds cleaved later.

With longer incubation times, peak II loses the A14 tyrosine to form peptide peak I. Peptide peak I, which is the amino end of the A and B chains, is not further degraded even after 1.5 h of incubation. Additionally, with longer incubation times, the peaks containing the carboxyl ends of the A and B chains are evidently further degraded. Only small amounts of insulin that had cleavage on the B chain with the A chain intact were observed.

Thus, human insulin protease may clip one site on the B chain and not the A chain, but the major insulin products have been cleaved at both the A and B chain peptide bonds with initial preference of A13-14 or A14-15 and B9-10, B13-14, and B16-17, with later cleavage at the B14-15, B24-25, A18-19, A19-20, and some B10-11 cleavage. In our earlier studies (15) also using native insulin but under different conditions and using rat insulin protease, we reported an initial cleavage site of insulin at B16-17.

Studies by Duckworth and co-workers (19, 53, 54) used radioactively labeled insulins and backtracked from the step on the sequenator at which the radioactivity came off to indicate that the peptide bond cleaved on the A or B chain. However, the remainder of the chain past the radioactivity and the other unlabeled A or B chain portion could not be determined. From these studies, they determined that rat insulin protease cleaved the bonds at A13-14, A14-15, B9-10, B10-11, B13-14, B14-15, B24-25, and B25-26.

Studies by Assoian and Tager (16) using [125I]iodotyrosyl-B1-insulin (125I-B1-insulin) reported that insulin protease in rat hepatocytes appeared to cleave insulin at three sites on the B chain (without stating the actual sites). In a separate study using a heterogeneous mixture of [125I]insulin, they concluded that the A13-14 position was cleaved by rat hepatocytes (17). These results were observed using the techniques of enzymatic digestion and electrophoretic mobilities. They also showed little change in the radioactive peak on gel filtration after incubation of 125I-B1-insulin with hepatocytes. This would agree with our finding of the A13-B19 insulin intermediate still eluting under the insulin peak.

Muir and co-workers (22), using synthetic [3H]Phe-B1-insulin and [3H]Gly-A1-insulins and chromatographic and electrophoretic techniques, deduced that rat insulin protease produced an insulin fragment that was most probably A1-13 connected by a disulfide bond to probably B1-9 and a minor component with a cleavage either between B10-11 or B11-12.

Using isolated rat hepatocytes and radioactively labeled insulin, Misbin et al. (18) found as a primary peak insulin missing the B19-30 portion of the molecule. This cleavage was
attributed to insulin protease. Several other studies have recently shown alterations in the radioactive insulin after incubation of radioactive insulin with various types of animal cells (25-28).

The bonds of insulin reported by these investigators to be cleaved when using intact animal cells or purified insulin protease correspond to several of the bonds that would be cleaved by the human insulin protease in this study to yield the intermediates observed. This indicates that the insulin protease in human and other animal models must be quite degrading enzyme shares homologies with an Escherichia coli protease (55) that has a high affinity for insulin. The bonds cleaved by insulin protease in these human tissues or other animal preparations thus far studied are not consistent with those cleaved by any other enzyme, such as the cathepsin family of enzymes B, D, or G, chymotrypsin, or other endopeptidases.

When viewing the insulin molecule with a linear diagram, the bond cleavages do not appear to have the specificity generally required of an enzyme. However, when viewing the three-dimensional structure of insulin by Hodgkins (56) (see Fig. 4), the sites of cleavage all appear to be in close proximity to leucine and tyrosine adjacent to the a-helical turns. This same Leu-Tyr area was noted with the degradation of glucagon by insulin protease (57). Therefore, insulin protease appears to cleave peptide bonds adjacent to the Leu-Tyr configurations in the three-dimensional structure. Additionally, it is interesting to note that it is this area of insulin which is involved in binding to the insulin receptor and that the phosphorylation of tyrosine on the receptor is a crucial factor in some of insulin's actions (8-12). In our present studies, when the purified enzyme is devoid of any lysosomal preparation, further degradation of insulin-intermediates to smaller peptides. Therefore, insulin protease appears to be generated very rapidly by insulin protease in the intact cell either while insulin is still bound to the receptor (42) or after endocytosis and exposure to the cytosol (13, 14, 60). Furthermore, in our present studies, when the purified enzyme is devoid of any lysosomal preparation, further degradation of intermediates to smaller peptides occurs with longer incubation times. This indicates that it is unlikely that lysosomal enzymes play a major role in the initial degradation of these intermediates to smaller peptides.

As to the significance of these intermediates and the smaller peptides and amino acids generated during the cleavage of the insulin bonds by insulin protease, it is tempting to postulate that they may serve as putative mediators of insulin action. In our preliminary studies, we have reported a stimulatory effect of the radioactively labeled intermediates on pyruvate dehydrogenase activity (45). Further studies are needed to ascertain if these intermediates from native insulin could serve in the capacity for short or longer action of insulin in modulating numerous metabolic events.

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