Human Red Blood Cell Insulin-degrading Enzyme and Rat Skeletal Muscle Insulin Protease Share Antigenic Sites and Generate Identical Products from Insulin*

(Received for publication, September 11, 1989)

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The mechanisms of cellular insulin degradation remain uncertain. Considerable evidence now exists that the primary cellular insulin-degrading activity is a metalloproteinase. Two similar degrading activities have been purified and characterized. Insulin protease has been purified from rat skeletal muscle and insulin-degrading enzyme from human red blood cells. Whereas the two degrading activities share a number of similar properties, significant differences have also been reported; and it is not at all established that they are the same enzyme. To examine this, we have compared antigenic and catalytic properties of the two enzymatic activities. Monoclonal antibodies against the red blood cell enzyme adsorb the skeletal muscle enzyme; and upon Western blots, the antibodies react with an identical 110-kDa protein. Immunoaffinity-purified enzymes from both red blood cells and skeletal muscle degrade \([125I]Iodoso(B26)insulin\) to the same products as seen with purified insulin protease and with intact liver and kidney. Chelator-treated muscle and red blood cell enzymes can be reactivated with either Mn\(^{2+}\) or Ca\(^{2+}\). Thus, insulin-degrading enzyme and insulin protease have similar properties. These results support the hypothesis that these activities reside in the same enzyme.

Insulin degradation is an integral part of the interaction of the hormone with insulin-sensitive tissues (1). Although binding to its specific receptor is the first step resulting ultimately in degradation of the hormone, the actual degradation occurs as a post-receptor process (2). Lysosomes have been implicated, but current evidence suggests that lysosomes are not involved, at least in the initiation of degradation which is prelysosomal (3, 4). Two major nonlysosomal proteolytic enzyme activities with specificity for insulin have been purified and characterized. The first of these, now generally termed insulin protease (5, 6), has been obtained primarily from muscle and has been implicated as physiologically important on the basis of general similarities between its properties and cellular degradation (7) and most recently because the degradation products generated from insulin by this enzyme are identical to cellular products (8, 9). The second enzymatic activity, termed insulin-degrading enzyme, has been purified from red blood cells (10). Again, the general properties of this enzyme are similar to cellular degradation properties, and its physiological importance has been shown by the fact that monoclonal antibodies to the enzyme partially inhibit cellular degradation of insulin (11). Whereas the described properties of insulin protease and insulin-degrading enzyme are similar, there are differences in the reported properties (including molecular weight, pH profile, metal requirement, and substrate specificity among others) (7); and it is not at all certain that these are the same enzyme.

To examine this question, we have used two properties, i.e. reaction with monoclonal antibodies and generation of degradation products, to compare these enzymes and conclude that they are identical or at least contain identical antigenic sites and generate identical products from insulin.

MATERIALS AND METHODS

\([125I]Iodoso(human insulin) (300-350 \text{ dpm})\) specifically labeled at Tyr\(^{70}\) was provided by Dr. Bruce Frank (Eli Lilly Research Laboratory) (12). Mouse monoclonal antibodies directed against purified human erythrocyte insulin-degrading enzyme have been described previously (11). Rabbit anti-mouse IgG was the generous gift of Dr. Martha Mellen- (Veterans Administration Medical Center, Omaha, NE). Acetomintril was HPLC- grade purchased from Fisher. All other chemicals were of at least reagent-grade. Freshly drawn human blood (heparinized tube) was centrifuged to sediment the red blood cells, and the plasma was removed. RBCs were washed three times with phosphate-buffered saline (PBS) (10 mM sodium phosphate, pH 7.4, 150 mM sodium chloride), and the resulting cell pellet was mixed with an equal volume of lysis buffer (50 mM HEPES, pH 7.6, 2% Triton X-100, 5 mM NaEDTA, 10 mM EGTA, 200 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 150 mM NaCl, 1 mg/ml bacitracin). After 30 min of incubation, the lysate was centrifuged to remove particulates and applied to the microtiter plate wells or diluted in PBS and then applied. Rat muscle insulin protease was purified by a modification of the previously described method (6) and used for the initial immunoprecipitation studies. A partially purified preparation was used for the product study. Briefly, hind leg muscle was homogenized in 0.35 M sucrose and centrifuged for 15 min at 13,000 \times g. The pellet was discarded, and the supernatant was centrifuged at 100,000 \times g for 1 h. The supernatant was then fractionated with ammonium sulfate, with the 30-60% fraction being dissolved in 20 mM sodium acetate, pH 6.2. Ammonium sulfate was removed by dialysis against 20 mM

*This work was supported in part by funds from the Veterans Administration and by National Institutes of Health Grants DK34926 and DK01393. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: HPLC, high performance liquid chromatography; RBCs, red blood cells; PBS, phosphate-buffered saline; HEPES, 5-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA [ethylenebis(oxyethylenenitrilo)tetraacetic acid].

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sodium acetate, pH 6.2; and partially purified enzyme was stored frozen until use. Microtiter plate wells were coated with rabbit antimusle IgG diluted to 11 μg/ml in PBS overnight at 4 °C. The wells were washed three times with PBS with 0.2% bovine serum albumin and then incubated with 25 μl of 10−4 M mouse monoclonal antibodies (9B12) to insulin-degrading enzyme for 4 h at 4 °C. The wells were washed three times with PBS, and either red blood cell lysate (20 μl of a 1:2, 1:5, or 1:10 dilution) or partially purified rat skeletal muscle insulin protease (1–10 μg) was added and incubated for 2.5 h at 4 °C. The enzyme preparation was removed, the wells were washed three times with PBS, and [125I]iodo(B26)insulin (2 × 10−8 M) in PBS was added and incubated at 37 °C for various times. The reaction was terminated by transferring the well contents to a test tube containing 50 μg of beef insulin, 50 μl of 20 mM sodium EDTA, and 0.23 g of urea. The volume was adjusted to 0.5 ml with 0.2 M ammonium phosphate, pH 8.5; and 50 mg each of sodium tetrathionate and sodium sulfite were added. After reaction for 20 min at room temperature, the mixture was stored at 4 °C until analysis by HPLC. Sulfitolized samples were analyzed by an HPLC method previously described (13) which separates the intact iodo-(B26)-B-chain from its proteolytic degradation products. Western blot analyses were performed as previously described (11) on enzyme purified from rat skeletal muscle by ammonium sulfate fractionation, ion-exchange chromatography, hydrophobic interaction chromatography, and molecular sieve chromatography (14). The purified enzyme was run on 7.5% polyacrylamide gels and transferred to nitrocellulose. The nitrocellulose was immunoblotted first with anti-insulin-degrading enzyme monoclonal antibody (9B12) and then with goat anti-mouse IgG antiant from the control tubes degraded 6.79 ± 0.38%, and the protein A-Sepharose beads were then assayed for insulin-degrading activity. The initial enzyme preparation degraded 8.86 ± 0.58% of [125I] iodo(A14)insulin to trichloroacetic acid-soluble material in 15 min (mean ± S.E. of three separate experiments). The supernatant from the control tubes degraded 6.79 ± 0.38%, and the control Sepharose beads degraded 1.39 ± 0.48% of the labeled insulin. In the monoclonal antibody samples, the supernatant degraded 1.54 ± 0.54%, and the protein A-Sepharose beads degraded 12.68 ± 0.37%. Thus, the majority of the degrading activity of our preparation of insulin protease was removed by treatment with the RBC insulin-degrading enzyme monoclonal antibody and could be assayed directly on the protein A beads. In other experiments (not shown) using larger amounts of antibody limited to Affi-Gel beads, all of the insulin-degrading activity of a muscle preparation could be bound to the antibody.

To test further whether the insulin protease was the same as human insulin-degrading enzyme, the two purified enzymes were electrophoresed on sodium dodecyl sulfate gel, transferred to a nitrocellulose filter, and incubated with the monoclonal antibody to insulin-degrading enzyme (Fig. 1). Both preparations contained a protein of M, ~ 110,000 which reacted with the antibody. This molecular weight is identical to that previously described for insulin-degrading enzyme (10, 11). Four different preparations of purified rat muscle enzyme were examined by Western blots including one purified by an insulin affinity column (6). All contained a 110-kDa band as exemplified in Fig. 1.

The next experiments were performed to examine the degr...
sulfitolized products from an experiment with less extensive degradation (~20% of the insulin degraded). A similar pattern is seen with both RBC insulin-degrading enzyme (upper) and muscle insulin protease (lower). Both profiles are identical to previously published profiles generated by hepatocytes, perfused kidney (15), renal tubule cells (16), and purified insulin protease (9). Thus, the products generated from [125I]iodo(B26)insulin by immunoprecipitated human RBC insulin-degrading enzyme, immunoprecipitated rat skeletal muscle insulin protease, purified muscle insulin protease, rat hepatocytes, and kidney cells are the same.

The experiments described above strongly suggest that these enzymatic activities are identical. A major difference in the reported properties of the two enzymes, however, is in their response to divalent cations. The muscle enzyme can be reactivated by Ca\(^{2+}\) after treatment with chelators, whereas the RBC enzyme apparently cannot. Both are reactivated by Mn\(^{2+}\). In order to compare directly the two preparations, monoclonal insulin-degrading enzyme antibody was added to RBC lysates and to the rat muscle 100,000 \(\times\) g supernatant, and the antigen-antibody complex was precipitated with Panisorbin. The precipitated enzyme complex was washed three times, and each supernatant and the pelleted enzyme complex was assayed for insulin-degrading activity. With the RBC lysate, 51% of the recovered activity was found in the pellet, and with the muscle supernatant, 54% was precipitated by Panisorbin. Each precipitate was then resuspended in 20 mM sodium acetate with 1 mM EDTA and dialyzed against this buffer overnight. Each pellet was assayed for insulin-degrading activity without and with various concentrations of Ca\(^{2+}\) and Mn\(^{2+}\) (Table I). As can be seen, Mn\(^{2+}\) reactivated both activities to the greater extent, with the maximal effect between 1.0 and 0.25 mM. Ca\(^{2+}\) also reactivated both enzyme preparations, with the greatest effect at 10 mM. At lower Ca\(^{2+}\) concentrations, the effect on the muscle enzyme was greater than the effect on the RBC enzyme. The experiment shown in representative of four separate experiments.

**DISCUSSION**

These data strongly suggest that RBC insulin-degrading enzyme and skeletal muscle insulin protease are the same enzyme. Monoclonal antibodies to RBC insulin-degrading enzyme bind the muscle enzyme, showing that they share an antigenic site. The immunoprecipitated enzymes produce the same products from [125I]iodo(B26)insulin. Both enzymes were shown to have an identical molecular weight on sodium dodecyl sulfate-polyacrylamide gels. Insulin protease has previously been shown (9) to cleave the B-chain of insulin in seven sites: four major, B9-B10, B13-B14, B16-B17, and B25-B26; and three minor, B10-B11, B14-B15, and B24-B25. The elution pattern of these B-chain peptides, after sulfitolysis of [125I]iodo(B26)insulin digested by purified insulin protease, as identical to those shown in Figs. 2 and 3 generated by enzyme immunoprecipitated by monoclonal antibodies to insulin-degrading enzyme from a crude muscle preparation and RBC lysates. These cleavage sites correspond to the peptides shown in Figs. 2 and 3 in the order of their elution: B25-B26, B24-B25, B16-B17, B13-B14, B14-B15, intact B-chain, B10-B11, and B9-B10. These peptides have also been found when insulin is degraded by intact hepatocytes (9), perfused kidney (15), and cultured renal tubule cells (16).

Previous studies (7) have shown a number of similarities between these two enzyme preparations, but have also shown some differences. The enzymatic activity has an essential sulfhydryl group (5, 6, 17), but also is inhibited by chelating agents (10, 18), suggesting that it is a sulfhydryl group-dependent metalloproteinase. The metal involved is not clear; but in our hands, insulin protease treated with EDTA can be reactivated by several metals including Zn\(^{2+}\), Mn\(^{2+}\), and Ca\(^{2+}\) under various conditions (18). RBC insulin-degrading enzyme has been reported not to be reactivated by Ca\(^{2+}\), and another group (19) has reported an inability to reactivate their chelator-treated preparation with any metal. In preliminary studies during this experiment, an enzyme preparation made with EDTA and assayed with Ca\(^{2+}\) was precipitated by the monoclonal antibody equally as well as the preparations made without EDTA. Thus, all of the insulin-degrading activities previously described are identical, or at least share an antigenic site.

In this study, we found that both RBC and muscle enzymes can be essentially totally inactivated by extensive treatment.

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with EDTA and that both can be reactivated by either Ca²⁺ or Mn²⁺. In addition, Mn²⁺ was more effective than Ca²⁺. At lower concentrations of Ca²⁺, the muscle enzyme was reactivated to a greater extent than the RBC enzyme, but the two were equally affected at 10 mM. Higher concentrations were not tested. In other studies (not shown), similar effects were obtained at both pH 6.5 and 8.5 as those shown in Table I at pH 7.5. The explanation for the small differences in divalent cation effects between muscle and RBC enzymes is not readily apparent. It is possible that differential amounts or types of modifiers could be present in the two tissues, such as the inhibitor recently described (20), which alter responsiveness. It is also of course possible that there could be differences between rat and human enzyme properties, and the presence of more than one similar insulin-degrading activity has not been totally excluded.

Extensive efforts were made to use the monoclonal antibody as an immunoaffinity reagent for purifying the enzyme for more extensive comparisons of its properties. Considerable difficulty, however, was encountered in displacing active enzyme from the antibody. Intermittent, partial success was obtained by using pH 11.0 buffers as reported (20); but even on the few occasions where this was successful, recovery of active enzyme was low, and the preparation rapidly lost activity. The effects of Ca²⁺ and Mn²⁺ in these preparations were similar to those shown in Table I, however.

This study does not resolve all of the previously reported discrepancies among the various insulin-degrading preparations (discussed in detail in Ref. 7). Some of these differences may be due to alterations during purification, variable copurification of endogenous inhibitors or modifiers, or copurification of other proteolytic activities. Nevertheless, this study allows us to conclude that the major insulin-degrading activity in muscle and RBCs is apparently identical and is probably the primary physiological mechanism for insulin degradation. We propose that the trivial name of insulin-degrading enzyme be used for this activity to emphasize its identity and to eliminate confusion in the literature.

Acknowledgments—The excellent technical assistance of Michael Mahoney and the secretarial assistance of Kimberly Dempsey are gratefully acknowledged.

REFERENCES
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