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Identification of Residues in the Insulin Molecule Important for Binding to Insulin-Degrading Enzyme†

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ABSTRACT: Insulin-degrading enzyme (IDE) hydrolyzes insulin at a limited number of sites. Although the positions of these cleavages are known, the residues of insulin important in its binding to IDE have not been defined. To this end, we have studied the binding of a variety of insulin analogues to the protease in a solid-phase binding assay using immunoimmobilized IDE. Since IDE binds insulin with 600-fold greater affinity than it does insulin-like growth factor I (25 nM and ~16 000 nM, respectively), the first set of analogues studied were hybrid molecules of insulin and IGF I. IGF I mutants [ins^{B1-17},17-70]IGF I, [Tyr^{55},Gln^{56}]IGF I, and [Phe^{23},Phe^{24},Tyr^{25}]IGF I have been synthesized and share the property of having insulin-like amino acids at positions corresponding to primary sites of cleavage of insulin by IDE. Whereas the first two exhibit affinities for IDE similar to that of wild type IGF I, the [Phe^{23},Phe^{24},Tyr^{25}]IGF I analogue has a 32-fold greater affinity for the immobilized enzyme. Replacement of Phe-23 by Ser eliminates this increase. Removal of the eight amino acid D-chain region of IGF I (which has been predicted to interfere with binding to the 23-25 region) results in a 25-fold increase in affinity for IDE, confirming the importance of residues 23-25 in the high-affinity recognition of IDE. A similar role for the corresponding (B24-26) residues of insulin is supported by the use of site-directed mutant and semisynthetic insulin analogues. Insulin mutants [B25-Asp]insulin and [B25-His]insulin display 16- and 20-fold decreases in IDE affinity versus wild-type insulin. Similar decreases in affinity are observed with the C-terminal truncation mutants [B1-24-His^{25}-NH_2]insulin and [B1-24-Leu^{25}-NH_2]insulin, but not [B1-24-Trp^{25}-NH_2]insulin and [B1-24-Tyr^{25}-NH_2]insulin. The truncated analogue with the lowest affinity for IDE ([B1-24-His^{25}-NH_2]insulin) has one of the highest affinities for the insulin receptor. Therefore, we have identified a region of the insulin molecule responsible for its high-affinity interaction with IDE. Although the same region has been implicated in the binding of insulin to its receptor, our data suggest that the structural determinants required for binding to receptor and IDE differ.

Insulin, insulin-like growth factor (IGF)[1] I, and insulin-like growth factor II constitute a family of hormones having a high degree of sequence and functional homology (Rinderknecht & Humbel, 1978a,b). Both insulin and IGF I have a number of growth-promoting effects, with the latter being identical with the growth-hormone-dependent somatomedin C (Klapper et al., 1983). A number of proteins have been described that are capable of binding with high affinity each member of the insulin/insulin-like growth factor (IGF) family. A unique cell-surface receptor exists for each of the peptides, which exhibits higher affinity for its specific ligand than for other membranes of the family [i.e., insulin receptor has the following order of affinities: insulin > IGF II > IGF I; for review, see Roth et al. (1988) and Czech (1989)]. In addition, serum binding proteins have been described that bind IGF I and IGF II but not insulin (Baxter & Martin, 1989). We have recently reported the molecular cloning and sequence analysis of another protein with differential affinities for insulin and the IGFs (Affholter et al., 1988). This protein—insulin-degrading enzyme (IDE)—is a cytosolic protease with a calculated molecular weight of 117 000 that shares structural and functional homology with bacterial protease III. In addition to hydrolyzing insulin in vitro, IDE appears to be re-

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[1] Abbreviations: IDE, insulin-degrading enzyme; IGF, insulin-like growth factor; EDTA, disodium ethylenediaminetetraacetate; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin.

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sponsible for the initial cleavage of internalized insulin by insulin-responsive cells [for review, see Duckworth and Kitabchi (1981) and Duckworth (1988)]. Questions have been raised regarding the possible role of this insulin degradation in both the termination and initiation of some insulin responses (Duckworth, 1988). Although the enzyme is inhibited by chelating agents (EDTA and 1,10-phenanthroline), sulfhydryl reactive agents [*N*-ethylmaleimide and *p*-(hydroxymercurio)benzoate], and bacitracin (Shii et al., 1986), specific inhibitors for the enzyme are not available. Furthermore, an analysis of the primary sequence of the enzyme reveals no significant homology with classical metalloproteases or cysteine proteases on which to base rational inhibitor design (Affholter et al., 1988). Although the major sites of cleavage of insulin by both mammalian (Assoian et al., 1981, 1982; Misbin et al., 1983; Muir et al., 1986; Duckworth et al., 1988; Stentz et al., 1989) and *Drosophila* (Duckworth et al., 1989) forms of the enzyme have been identified and shown to be nearly identical, little is known about the regions of the insulin molecule that are responsible for high-affinity binding to IDE. Identification of such residues is important in understanding the biochemical interactions of insulin with IDE and should facilitate the design of protease-resistant insulin analogues.

Recently, a number of groups have sought to identify the structural domains of insulin and IGF I/II responsible for binding of the peptides to both serum binding proteins (DeVroede et al., 1985; Joshi et al., 1985; Bayne et al., 1988a) and receptors (Tager et al., 1980; Cara et al., 1988; Cascieri et al., 1988; Cascieri et al., 1989). In the most recent studies, a synthetic human IGF I gene was constructed and placed in a yeast expression system (Bayne et al., 1988b). Site-specific mutations were introduced into the gene such that a number of residues in IGF I were altered to the corresponding amino acids of insulin, making the analogues more "insulin-like" in sequence. These studies have defined an important role for amino acids B3–4/B15–16 in the interaction of IGF I with serum binding proteins (Bayne et al., 1988a). In contrast, the B24–26 region has been shown to be critical to the high-affinity interaction of both insulin (Tager & Nakagawa, 1987) and IGF I (Cascieri et al., 1988) with their receptors. In the present study, we have used the same IGF I analogues and a number of insulin analogues in conjunction with a unique solid-phase "enzyme binding assay" to define the regions of insulin/IGF I responsible for high-affinity binding of the hormone to IDE.

Materials and Methods

Iodination of Insulin. Iodination of insulin was by the Iodogen method (Pierce). Borosilicate test tubes were coated with Iodogen reagent and washed with 500 μL of 0.5 M sodium phosphate, pH 7.4. Fifty microliters of 0.5 M sodium phosphate, pH 7.4, containing 2 μg of insulin was added to each tube followed by the addition of 1 mCi of $Na^{125}I$ (carrier free, Amersham). Samples were incubated at room temperature for 10 min. Reactions were stopped by the addition of 200 μL of column equilibration buffer (20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 2.5 mg/mL BSA) containing 10 mg/mL KI and 10 mg/mL cytochrome *c*. Iodinated hormone was separated from unincorporated ^{125}I by elution through a PD-10 column (Pharmacia) preequilibrated with column equilibration buffer. This method typically resulted in a labeled product with specific activity of 150–300 Ci/g.

IGF I Mutants. Mutagenesis and expression from the synthetic IGF I gene have been described previously (Bayne et al., 1987). Bayne et al. (1988b) have shown that expression of IGF I in yeast (1-L cultures) results in production of 200–400-μg quantities of fully active peptide. IGF analogues were homogeneous as determined by SDS–polyacrylamide gel electrophoresis and the mass was quantitated by amino acid analysis.

Insulin Mutants. Mutagenesis and expression of the single-chain insulin precursor cDNA to generate the [B25-Asp]- and [B25-His]insulin used in this study has also been described (Brange et al., 1989). The semisynthesis of insulin lacking the carboxy tail of the B-chain and containing amino acid substitutions at position B25 was described recently by Brandenburg et al. (1988).

Red Cell Lysates. Human blood and/or packed red blood cells (400 mL) were obtained from the Stanford University Blood Bank, pelleted by centrifugation at 3000*g* and 4 °C for 15 min, and the pellet was resuspended in an equal volume of lysis buffer (50 7M Hepes, pH 7.6, 150 mM NaCl, 2% Triton X-100, 5 mM EDTA, 1 mM PMSF, and 1 mg/mL bacitracin) before freezing at −20 °C. Prior to use in binding assays, aliquots of the lysate were thawed on ice.

IDE Binding Assays. Poly(vinyl chloride) microtiter plates (Falcon, 96-well plates) were coated with 50 μL of 50 μg/mL affinity purified rabbit antimouse IgG (Pel-Freeze) in 20 mM $NaHCO_3$, pH 9.6, at room temperature for 1–2 h. Wells were washed with cold WG+ (50 mM Hepes, pH 7.6, 150 mM NaCl, 0.2% BSA, and 0.1% Triton X-100) and coated with anti-IDE monoclonal antibody 9B12 (Shii & Roth, 1986) at 5 μg/mL in WG+ as above. Wells were washed once more, placed on ice, overlayed with 40 μL of red cell lysate, and incubated overnight at 4 °C. After two washes, wells were overlayed with 30 μL of ice-cold WG+ containing 20000 cpm of [^{125}I]insulin (~1 nM) and appropriate concentrations of unlabeled hormone or analogue. Wells were incubated at 4 °C for 4–6 h and washed twice with WG+ and the number of counts bound was determined by using a Beckman Model 5500 γ counter. Typical assays gave 1000–3000 cpm of [^{125}I]insulin specifically bound to immobilized IDE whereas nonspecifically bound [^{125}I]insulin was typically <150 cpm.

Receptor Binding Assays. Receptor binding assays were essentially identical with the IDE binding assays except that the second antibody (at 1 μg/mL) was antiinsulin receptor monoclonal antibody 29B4 (Morgan & Roth, 1986). To immunoimmobilize the insulin receptor, antibody-coated wells were overlayed with fresh CHO-T (Chinese hamster ovary cells overexpressing the human insulin receptor at ~1×10^6 receptors per cell; Ellis et al., 1986) lysate and incubated overnight at 4 °C. CHO-T lysates were prepared by lysing confluent 100-mm plates of CHO-T in 3 mL of lysis buffer minus EDTA, PMSF, and bacitracin. Nuclei were removed by centrifuging at 10000*g* for 10 min at 4 °C. Lysate was stored on ice until needed. Typical assays resulted in specific binding of 1500–4000 cpm.

Results

IGF I Analogues. Because IDE has negligible enzymatic activity below 10 °C, it is possible to perform a receptor-like binding assay for the enzyme in which the affinities of unlabeled insulin, insulin-like growth factors, and their analogues are measured directly by their ability to compete with [^{125}I]insulin for binding to the protease. Using this assay, we have determined the binding properties of insulin, IGF I, and IGF II (Figure 1). The concentration of a given peptide necessary to inhibit 50% of the specific binding of [^{125}I]insulin to the immobilized enzyme was defined as the IC_{50}. This constant differs slightly from the traditional IC_{50} values for proteolytic enzymes in that it reflects only the association and

Table I: Binding of IGF I and Insulin Analogues to Human Insulin-Degrading Enzyme (IDE), Insulin Receptor (IR), IGF I Receptor (IGF I-R), Serum Binding Proteins (BP), and Rat IGF II Receptor (IGF II-R)

peptide	IC_{50} IDE[a] (μM)	IR[b] (μM)	IGF I-R (nM)	IGF II-R (μM)	BP (μM)	ref
IGF I	16	2.8 ± 1	5.6 ± 0.8	0.3 ± 0.2	0.48 ± 0.02	Cascieri, 1988
IGF II	0.56	0.063				
insulin	0.025	0.002 ± 0.0007				
B-chain mutant	4.0 ± 0.9	0.7 ± 0.3	12 ± 1	≫5	≫1000	Bayne, 1988a
[Y^{55},Q^{56}]IGF I	25 ± 12	1.7 ± 0.1	4.7 ± 1.1	0.06 ± 0.04	0.35 ± 0.01	Cascieri, 1989
[F^{23},F^{24},Y^{25}]IGF I	0.5 ± 0.1	1.5 ± 0.3	7.7 ± 0.8	0.1 ± 0.04	1.2 ± 0.4	Cascieri, 1988
[S^{23},F^{24},Y^{25}]IFG I	31 ± 12	>2	~78	>9	~5.8	Cascieri, 1988
[1–62]IGF	0.63 ± 0.25	0.7 ± 0.3	6.4 ± 1.3	0.4 ± 0.1	0.17 ± 0.01	Bayne, 1989
[L24,1–62]IGF I	0.16 ± 0.06	13 ± 1	490 ± 5	0.3 ± 0.03	0.27 ± 0.01	Cascieri, 1988
[Asp^{B25}]insulin	0.4 ± 0.2	0.32 ± 0.07				
[His^{B25}]insulin	0.5 ± 0.2	0.0079 ± 0.0016				
[B1–24-$His4^{B25}$-NH_2]insulin	0.25 ± 0.15	0.0016 ± 0.0004				
[B1–24-Leu^{B25}-NH_2]insulin	0.18 ± 0.05	0.013 ± 0.005				
[B1–24-Trp^{B25}-NH_2]insulin	0.079 ± 0.021	0.013 ± 0.005				
[B1–24-Tyr^{B25}-NH_2]insulin	0.032	0.018	0.0013 ± 0.0007			

[a] Mean ± SD, $n \geq 3$ in all cases. Since binding is measured under temperatures at which no degrdn. occurs (see text), IC_{50} here refers only to inhibition of binding, not enzyme activity. [b] For the IGF I related peptides, the IC_{50} values for binding to insulin, IGF I, and IGF II receptors and serum binding proteins are taken from the indicated references. Binding data for the insulin analogues was determined directly.

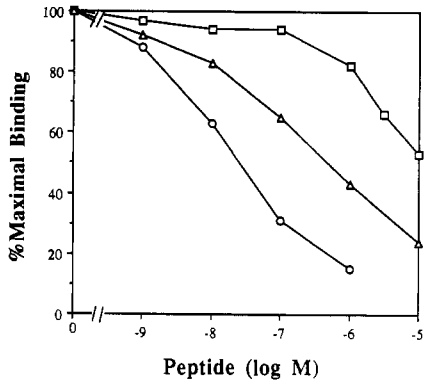

FIGURE 1: Inhibition of [^{125}I]insulin binding to immunoimmobilized IDE by unlabeled insulin (○), IGF I (□), and IGF II (△). Data are expressed as the percent of maximal specific binding of [^{125}I]insulin determined in the absence of added peptide. Each point represents the mean of at least five triplicate determinations.

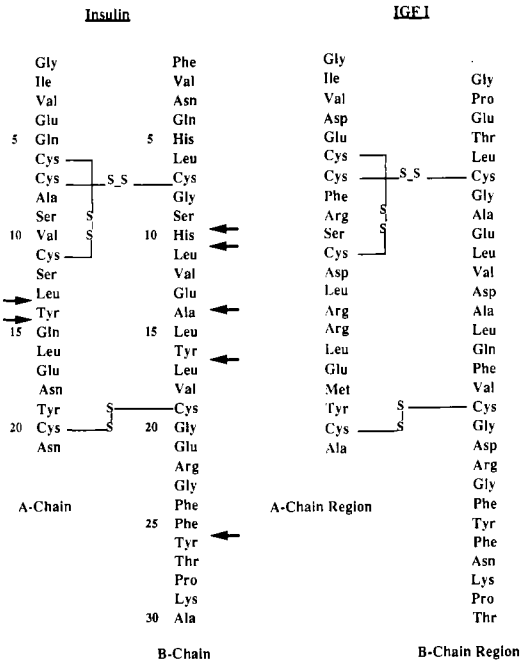

FIGURE 2: Primary structure of insulin and the corresponding A- and B-chain regions of IGF I. Arrows indicate major sites of cleavage of insulin by IDE (Duckworth, 1988). (Note: The C- and D-chain regions of IGF I are not shown in this figure.)

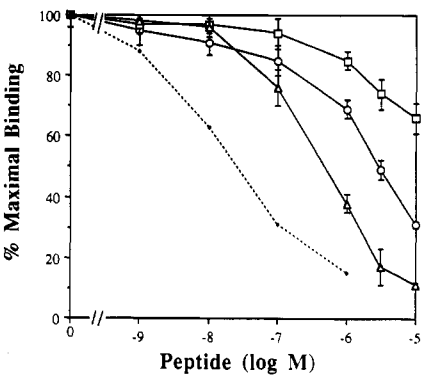

FIGURE 3: Inhibition of [^{125}I]insulin binding to immunoimmobilized IDE by unlabeled [ins^{B1-17},17–70]IGF I (○), [Tyr^{55},Gln^{56}]IGF I (□), and [Phe^{23},Phe^{24},Tyr^{25}]IGF I (△). Competition by unlabeled insulin (---) is shown for comparison. Data are expressed as the percent of maximal specific binding of [^{125}I]insulin determined in the absence of added peptide. Each point represents the mean ± SD for at least three identical determinations.

dissociation of substrate and competing peptide exclusive of the dissociation of hydrolytic products. Figure 1 shows that whereas IDE has an IC_{50} for insulin of 25 nM, its IC_{50} for IGF II and IGF I are 0.56 and 16 μM, respectively.

To investigate the molecular basis for this large range of affinities, we tested a series of IGF I mutants substituted with insulin-like residues (Bayne et al., 1988a, 1989; Cascieri et al., 1988, 1989) at positions corresponding to major cleavage sites of insulin by IDE (i.e., A13-Leu–A14-Tyr, A14-Tyr–A15-Gln, B9-Ser–B10-His, B14-Glu–B15-Ala, B16-Tyr–B17-Leu, and B26-Phe–B27-Tyr; Figure 2). The B-chain mutant[2] ([ins^{B1-17},17–70]IGF 2) replaces the first 16 amino acids of IGF I (corresponding to insulin B2–17) with the first 17 amino acids of insulin. The resulting peptide contains three of the major insulin cleavage sites. The [Phe^{23},Phe^{24},Tyr^{25}]IGF I mutant replaces the Tyr^{24}-Phe^{25} dipeptide of IGF I with the Phe-Tyr sequence of insulin. The [Tyr^{55},Gln^{56}]IGF I mutant replaces the Arg^{55}-Arg^{56} of IGF I with the corresponding Tyr-Gln of insulin such that A13–A15 region (containing two cleavage sites) is identical with that of insulin. The ability

[2] Numbering of residues in IGF I analogue follows the IGF I numbering system. Numbering of residues in insulin analogues follows the insulin B-chain numbering system. Therefore, positions 23–25 of IGF I align with residues B24–26 of insulin.

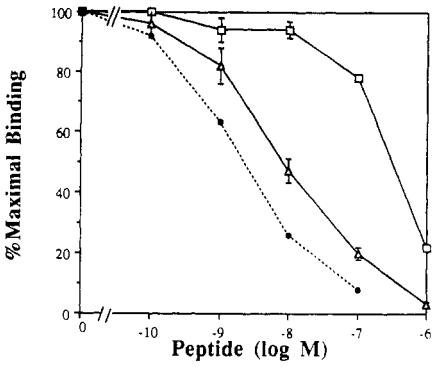

FIGURE 4: Inhibition of [^{125}I]insulin binding to immunoimmobilized insulin receptor by unlabeled [Asp^{B25}]insulin (□) and [His^{B25}]insulin (△). Competition by unlabeled insulin (---) is shown for comparison. Data are expressed as described in Figure 3 legend.

of these analogues to inhibit binding of insulin to IDE is shown in Figure 3. The B-chain mutant exhibits an IC_{50} of 4 μM, 4-fold greater than the affinity of wild-type IGF I (see Table I for comparisons), indicating a relatively small contribution of this region to the high-affinity interaction of insulin with IDE. This analogue also shows an identical 4-fold increase in affinity for the insulin receptor (Table I; Bayne et al., 1988a). The [Tyr^{55},Gln^{56}]IGF I analogue exhibits an affinity for IDE identical with that of wild-type IGF I, indicating that this region of the A-chain does not contribute significantly to the high-affinity interaction between insulin and IDE. The analogue [Phe^{23},Phe^{24},Tyr^{25}]IGF I, however, has an IC_{50} of 0.5 μM (Figure 3), a 32-fold increase in affinity for IDE, while maintaining its potency at both insulin and IGF I receptors (Cascieri et al., 1988). These data suggest that the aromatic B24–26 region of insulin is critical for high-affinity binding to IDE.

Loss of aromaticity at position 23 of IGF I by substitution of Ser for Phe (Table I) completely eliminates the increase in IDE affinity gained by inversion of the order of the Phe and Tyr residues. This result is consistent both with the importance of residues 23–25 (B24–26) in binding to IDE and with an apparent requirement for hydrophobic amino acids in this region.

According to Blundel et al.'s (1983) model of IGF I tertiary structure, the eight amino acid D-chain peptide will fold in such a way as to obstruct access to the 23–25 region of IGF I. Removal of the D-chain should result in an increase in affinity toward any macromolecule that recognizes the previously obstructed domain. In fact, desoctapeptide-IGF I ([1–62]IGFI) displays only a modest increase in affinity for the insulin receptors and no change in affinity for IGF I receptors (Bayne et al., 1989) but exhibits a 25-fold increase in affinity (IC_{50} = 0.63 μM) for IDE over IGF I (Table I). It has also been reported that replacement of the aromatic Tyr^{24} residue in IGF I or desoctapeptide-IGF I with Leu results in a sharp decrease in affinity for both IGF I and insulin receptors but not serum binding proteins (Cascieri et al., 1988). Since IGF I and insulin appear to recognize a hydrophobic/aromatic pocket in the binding cleft of IDE, we tested the importance of aromaticity at position 24 using the [Leu^{24},1–62]IGF I analogue. This peptide exhibits the highest affinity of all IGF I analogues tested to date (Table I), suggesting that extended hydrophobic side chains at position 24 may enhance the affinity of insulin/IGF I analogues for IDE.

Insulin Analogues. To confirm the importance of the B24–26 region in the binding of insulin to IDE, the two site-specific mutants of insulin were assayed for their ability to bind the immobilized enzyme. Substitution of position B25

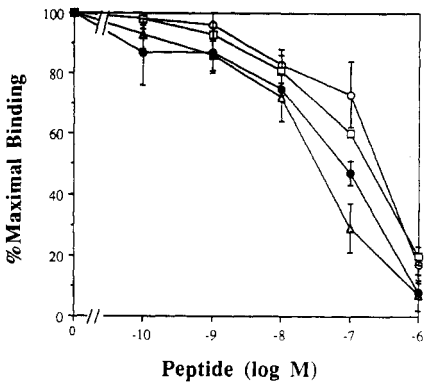

FIGURE 5: Inhibition of [^{125}I]insulin binding to immunoimmobilized IDE by unlabeled [B1–24-His^{25}-NH_2]- (○), [B1–24-Leu^{25}-NH_2]- (□), [B1–24-Tyr^{25}-NH_2]- (△), and [B1–24-Trp^{25}-NH_2]insulin (●). Data are expressed as described in Figure 3 legend.

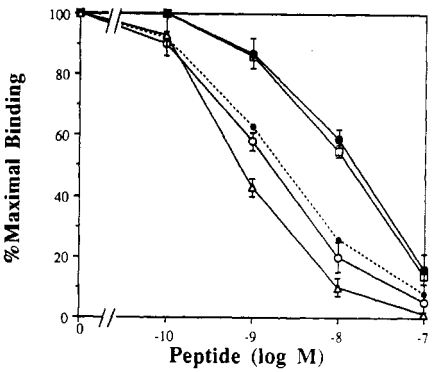

FIGURE 6: Inhibition of [^{125}I]insulin binding to immunoimmobilized insulin receptor by unlabeled [B1–24-His^{25}-NH_2]- (○), [B1–24-Leu^{25}-NH_2]- (□), [B1–24-Tyr^{25}-NH_2]- (△), and [B1–24-Trp^{25}-NH_2]insulin (●). Competition by unlabeled insulin (---) is shown for comparison. Data are expressed as described in Figure 3 legend.

(Phe) with the nonaromatic nonhydrophobic amino acids Asp or His results in respective 16- and 20-fold decreases in affinity (Table I). Despite such similar affinities for IDE, however, the ability of these analogues to bind to the insulin receptor differs significantly as shown in Figure 4. Whereas the B25-His analogue exhibits an IC_{50} at the insulin receptor of 7.9 nM (4-fold lower than insulin), the B25-Asp mutant has a value 40-fold lower (IC_{50} = 320 nM).

A set of insulin B-chain truncation mutants were also tested for their ability to compete with [^{125}I]insulin binding to immobilized protease. These semisynthetic insulin analogues were constructed as described previously (Brandenburg et al., 1988) and combine a deletion of the final five amino acids of the B-chain with a substitution of either His, Leu, Trp, or Tyr in the resulting B25 C-terminal position. As shown in Figure 5 and summarized in Table I, the B25-Trp and B25-Tyr mutants have affinities for IDE similar to that of wild-type insulin whereas the B25-Leu and B25-His analogues exhibit decreases in affinity of 7- and 10-fold, respectively. Interestingly, the analogue (B25-His) with 10-fold lower affinity for IDE than insulin displays an affinity for the insulin receptor (IC_{50} = 1.6 nM) near that of insulin (Figure 6).

Discussion

We have identified regions of the insulin molecule important in its interaction with insulin-degrading enzyme. A unique solid-phase assay was used to determine the ability of insulin, IGF I, IGF II, and various analogues of insulin and IGF I to compete with [^{125}I]insulin for binding to immunoimmobilized IDE. Both the magnitude and order of affinities of the three wild-type hormones for the immobilized enzyme agree well

with their reported susceptibilities to proteolysis by the enzyme (Misbin et al., 1983; Roth et al., 1984; Misbin & Almira, 1989). For example, the IC_{50} for insulin determined by this assay (25 nM) is close to the Michaelis–Menten K_m determined previously (20–100 nM) for the hydrolysis of insulin (Duckworth, 1988). In addition, the IC_{50} for IGF II and IGF I of 0.56 and 16 μM, respectively, agrees with earlier studies showing that IDE degrades insulin best, IGF II next, and IGF I at a very low rate (Misbin et al., 1983; Roth et al., 1984; Misbin & Almira, 1989). These results are consistent with the hypothesis that the ability of IDE to bind a particular analogue will in part determine its ability to hydrolyze that analogue. However, it is possible that some analogues which bind tightly to IDE may not be degraded by the enzyme.

We then set out to investigate the molecular basis for the 600-fold difference in affinity of the protease for insulin and IGF I. We postulated that residues involved in the binding of insulin to IDE would be located at or near the sites at which insulin is hydrolyzed by the enzyme. For this reason, IGF I analogues [ins^{B1-17},17–70]IGF I (Bayne et al., 1988a), [Tyr^{55},Gln^{56}]IGF I (Cascieri et al., 1989), and [Phe^{23},Phe^{24},Tyr^{25}]IGF I (Cascieri et al., 1988), each of which contains insulin-like residues at positions corresponding to reported sites of cleavage of insulin by IDE (Figure 2) (Muir et al., 1986; Duckworth et al., 1988; Stentz et al., 1989), were assayed for their abilities to compete with [^{125}I]insulin for binding to IDE. Of these peptides, only the [Phe^{23},Phe^{24},Tyr^{25}]IGF I mutant exhibited a substantial increase in affinity (32-fold) for the immobilized protease, suggesting that the Phe-Tyr-Phe residues (positions 23–25) of IGF I and the B24–26 Phe-Phe-Tyr of insulin may interact with a hydrophobic binding pocket at or near the active site of IDE. Since this analogue represents a simple inversion of the Tyr-Phe sequence in the context of a native IGF I backbone and does not significantly effect binding to other proteins, the increase in affinity for IDE reflects some intrinsic property of the insulin/IGF I binding site of IDE.

The B24–26 (23–25 in IGF I) region has also been implicated in the binding of insulin and IGF I to their receptors (Tager et al., 1980; Tager & Nakagawa, 1987; Brandenburg et al., 1988; Cascieri et al., 1988; Cascieri et al., 1989). The Tyr^{24} and Phe^{25} residues of IGF I, however, appear interchangeable with respect to receptor binding since [Phe^{23},Phe^{24},Tyr^{25}]IGF I exhibits the same affinity as IGF I (which has the sequence Phe-Tyr-Phe) at both IGF I and insulin receptors (Table I). This interchangeability is not observed in the binding of these analogues to the protease (Figure 3).

A model can be constructed in which each of these three amino acid side chains interacts with a slightly different hydrophobic subsite of the IDE binding pocket (S1, S2, and S3 corresponding to IGF I positions 23, 24, and 25, respectively) in which S3 is somewhat more hydrophilic than S1 and S2, allowing for stabilization of the tyrosine hydroxyl when the residue resides in position 25 but not in position 24 (Figure 7). This model would predict that replacement of any of these amino acids with nonhydrophobic residues would significantly diminish the ability of such analogues to bind IDE and that replacement of Tyr-24 of IGF I with a more hydrophobic residue should result in a peptide with increased binding affinity for IDE. The first of these predictions was tested by using an IGF I peptide that preserved the beneficial Phe-Tyr inversion but also incorporated the substitution of a Ser residue at position 23. The effect of this change was to eliminate the increase in affinity gained by reversing the Tyr-Phe sequence.

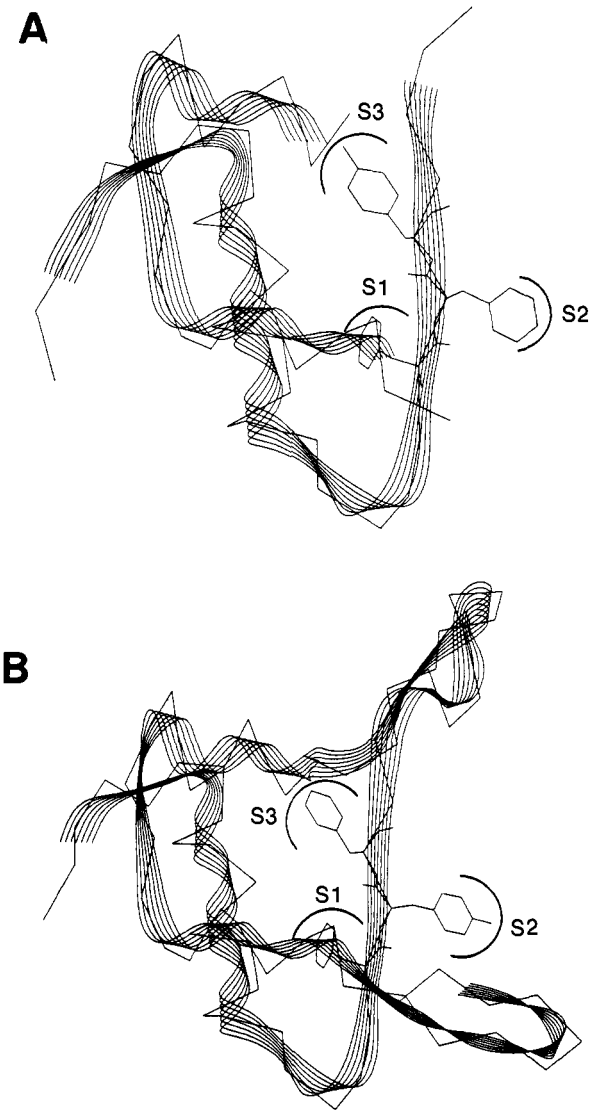

FIGURE 7: Comparison of the crystal structure of porcine insulin (Dodson et al., 1974) (A) with the proposed structural model of IGF I (Blundel et al., 1983) (B). In the insulin structure, side chains of amino acid residues Phe^{24}, Phe^{25}, and Tyr^{26} are shown. In the human IGF I structure, side chains of residues Phe^{23}, Tyr^{24}, and Phe^{25} are shown. Putative binding subsites S1, S2, and S3 of IDE are shown schematically.

The obvious explanation for this result is that hydrophilic amino acids (at position 23) are not compatible with high-affinity recognition by IDE. Alternatively, substitutions at position 23 may cause destabilization of the tertiary structure of IGF I as has been suggested (Cascieri et al., 1988).

Blundell et al. (1983) have proposed a model for the tertiary structure of IGF I based on the crystal structure of insulin. According to this model (Figure 7), any protein that interacts with the B24–26 region of insulin will exhibit lower affinity for IGF I (residues 23–25) due to steric interference by D-chain residues 6–8 (IGF I residues 63–70). Conversely, removal of the IGF I D-chain residues will result in an increase in affinity for such proteins. Based on this prediction we have examined the binding of desoctapeptide-IGF I (which lacks the D-chain region) to IDE and find that it does exhibit the expected increase in affinity (25-fold) (Table I), thus confirming by an independent approach the importance of the 23–25 (B24–26) region in conferring high affinity for IDE. Interestingly, the same analogue does not exhibit the expected increase in affinity for insulin receptor or decrease in affinity for IGF I receptor (Table I). Although the same regions of

insulin and IGF I appear to be involved in binding to both IDE and receptors, these results suggest a fundamental difference between the mechanism by which the protease and the receptors recognize these regions.

Taken together, these results suggest that the higher affinity of insulin than IGF I for IDE may arise from two independent sources as can be seen in Figure 7. One contributing factor appears to be the difference in primary sequence between positions B25–26 (Phe-Tyr) of insulin and the corresponding positions 24–25 (Tyr-Phe) of IGF I. The second is the steric interference caused by the presence of D-chain residues in IGF I but not in insulin.

To further characterize the peptide binding site of IDE, we tested another desoctapeptide IGF I analogue that, in addition to lacking the D-chain region, incorporates the substitution of Leu for Tyr at position 24. This analogue exhibits the highest affinity for IDE of the IGF analogues tested (Table I), suggesting that the highly extended hydrophobic side chain of leucine is more compatible with the S2 subsite than is the hydroxylated aromatic side chain of tyrosine. That substitution of a nonaromatic, hydrophobic side chain in this position results in an increase in affinity for IDE again suggests a significant difference between the mechanism of binding of IGF to IDE versus binding to receptor. In the latter case, substitution of Tyr-24 with a nonaromatic residue results in a marked decrease in affinity (Cascieri et al., 1988).

To confirm the importance of the B24–26 region in facilitating binding of insulin to IDE, we determined the capacity of insulin analogues substituted at position B25 by either Asp or His to bind IDE (Brange et al., 1988). Both mutants exhibit marked decreases in affinity (16- and 20-fold, respectively) for IDE (Table I). Since the same region is involved in binding of insulin to its receptor, we tested these analogues for their ability to bind insulin receptor and found that the affinity of the His mutant was only slightly diminished (4-fold versus wild type) whereas the Asp mutant exhibited a 160-fold reduction in affinity (Figure 4). These results not only confirm the role of residues B24–B26 but, in conjunction with the Leu-24 results just described, suggest that it may be possible to preserve receptor binding capacity while altering protease affinity—a feature absolutely essential for the future development of protease-resistant insulin analogues.

A set of semisynthetic insulin analogues have also been reported that lack the final five amino acids of the B-chain and incorporate amino acid changes at the C-terminal B25 position (Brandenburg et al., 1988). These analogues are interesting because they exhibit affinities for the insulin receptor (Figure 6) and biopotencies near that of wild-type insulin (from ~20–300% wild type). Given the extreme aromatic/hydrophobic composition of the B24–26 region of insulin, it is not surprising that aromatic substitutions [such as Tyr (wt) or Trp] at B25 result in analogues having affinities close to that of insulin. Substitution by a hydrophilic (His) residue, however, results in a 10-fold decrease in affinity for IDE (Figure 5). Interestingly, the magnitude of this decrease is less than that observed with the untruncated [B25-His]-insulin, perhaps suggesting that removal of the B26-Phe residue and the carboxy tail increases the mobility of the B25-His side chain allowing it to assume a more thermodynamically favorable orientation with respect to the hydrophobic binding pocket (S2) of IDE. In fact, Brandenburg et al. (1988) have shown that removal of the C-terminal tail of the B-chain increases the flexibility of the B25 (Phe) side chain. In addition, stabilization of the amide group by the "hydrophilic component" of S3 may also contribute to a less-than-predicted loss of affinity. Since there are only two amino acids to occupy a binding pocket of three subsites, however, it is unclear whether the C-terminal residues in this series of peptides interact with the S2 or S3 subsites of IDE. Stabilization of the amide by S3 may also result in a less-than-optimal positioning of the Leu side chain with respect to subsite S2 (or that the Leu side chain is actually occupying the S3 subsite), resulting in a truncated B25-Leu analogue with somewhat lower affinity than insulin for IDE. In either case, the B25-Leu data confirm the IGF I analogue data, suggesting that nonaromatic, hydrophobic amino acids at position B25 are compatible with high-affinity binding to IDE.

In summary, the present study describes the use of a solid-phase binding assay to characterize the residues of insulin and IGF I required for high-affinity binding to an insulin-specific protease. These results further characterize the insulin and IGF molecules with respect to their interactions with other proteins and provide the first molecular insights into the biochemical interaction between insulin and IDE. These insights may facilitate the design of protease-resistant insulin analogues and peptide-based inhibitors of insulin degrading enzyme, which will be useful in further elucidating the role of IDE in insulin and IGF action.

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Specificity and Orientation of (Iodoacetamido)proxyl Spin-Labeled Myosin Subfragment 1 Decorating Muscle Fibers: Localization of Protein-Bound Spin Labels Using SDS-PAGE†

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ABSTRACT: The nitroxide spin label (iodoacetamido)proxyl (IPSL) was specifically and rigidly attached to sulfhydryl 1 (SH1) on myosin subfragment 1 (S1). The specificity of this label for SH1 was demonstrated by using a technique where the spin label is localized on the electrophoresis-isolated proteolytic fragments of myosin using electron paramagnetic resonance (EPR). Studies of the rigidity of the probe on SH1 indicate that the IPSL is immobilized on the surface of S1 in the presence and absence of the nucleotides MgADP or MgATP. The EPR spectrum of muscle fibers decorated with IPSL–S1 shows that the IPSL–S1 rotates from its orientation in rigor upon binding MgADP. The angular displacement due to nucleotide binding is larger than that detected with the (maleimido)tempo spin label [Ajtai, K., French, A. R., & Burghardt, T. P. (1989) *Biophys. J. 56*, 535–541], demonstrating that the IPSL is oriented on the myosin cross-bridge in a manner that is favorable for detecting cross-bridge rotation during the rigor to MgADP state transition.

Orientation changes of fluorescent or spin probes attached to reactive side chains on the myosin cross-bridge demonstrate the ability of the cross-bridge to maintain differing orientations while bound to the actin filament in muscle fibers. The orientation of either type of probe is characterized by a molecular coordinate frame fixed in the probe. The orientation of the probe-fixed frame can be detected (with varying degrees of angular resolution) by fluorescence polarization or electron paramagnetic resonance (EPR). For some time now we have been measuring the orientation of a variety of probes as a function of time and/or the physiological state of the muscle. We find that the probes have differing sensitivities to the cross-bridge rotation consistent with the view that some of the

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