

Regulation of Multicatalytic Enzyme Activity by Insulin and the Insulin-Degrading Enzyme*

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ABSTRACT

The insulin-degrading enzyme (IDE) plays an important role in the cellular metabolism of insulin. Recent studies have also suggested a regulatory role for this protein in controlling the activity of cytoplasmic protein complexes, including the proteasome [multicatalytic proteinase (MCP)] and the glucocorticoid and androgen receptors. Binding of IDE to these complexes increases their activity, whereas the addition of substrates for IDE inhibits activity. This provides a potential mechanism of action for internalized insulin and other IDE substrates in the control of protein turnover. To examine further the interactions, partially purified IDE-MCP complex was treated with EDTA or EGTA, and activity was mea-

sured in the absence and presence of various divalent cations (Ca^{2+} , Mn^{2+} , Co^{2+} , and Zn^{2+}) and insulin. EDTA treatment reduced MCP activity and eliminated the effect of insulin on the complex. Divalent cations partially or completely restored MCP activity, but did not restore the effect of insulin. EGTA treatment had a lesser effect on MCP activity, but abolished insulin inhibition of activity. Divalent cations restored the insulin effect. Inhibitors of IDE also blocked the insulin effect on MCP activity, as did treatment with SDS. These findings suggest that conformational changes in the complex may play a role in the insulin control of MCP activity. (*Endocrinology* **139**: 4061–4066, 1998)

INSULIN-DEGRADING enzyme (IDE; EC 3.4.24.56), is an intracellular protein with binding, regulatory, and degradative functions. *In vitro*, its most characteristic function is proteolytic activity, with a relatively high specificity for insulin, thus leading to its name (1). Although insulin is the substrate with the highest affinity, IDE also degrades a number of other peptides and hormones, including glucagon (2), insulin-like growth factor II (3), atrial natriuretic peptide (4), and transforming growth factor- α (5). It is not a general proteinase, because it does not degrade a wide variety of other similarly sized peptides, and no specific amino acid sequence is required for proteolysis (2). These findings have led to the conclusion that IDE recognizes a three-dimensional structure for binding and degradation (5, 6). The requirement for a specific ligand structure is reflected in the binding properties of IDE. This protein binds proinsulin, insulin-like growth factor I, and epidermal growth factor with high affinities, but has little degrading activity toward them (7). In fact, IDE was first identified in *Drosophila* by its binding properties and was considered a new intracellular growth factor receptor (8).

The regulatory properties of IDE are a relatively new finding. IDE associates with and activates the cytosolic glucocorticoid and androgen receptors (9, 10). IDE also complexes with the proteasome and regulates its activity (11). Substrates that bind to IDE, including insulin and the various growth factors, inhibit proteasome activity when IDE is complexed

with multicatalytic proteinase (MCP), but not after separation of the two proteins (12, 13). Furthermore, insulin inhibits proteasome activity toward an artificial substrate in intact cells (14). This has led to the hypothesis that intracellular actions of insulin and other growth factors on cellular protein turnover are due to interactions with IDE and inhibition of proteolysis by the proteasome.

IDE is a metalloenzyme containing Zn^{2+} and possibly Mn^{2+} (15), and its degradative activity requires one or more divalent cations (7). In addition to Zn^{2+} and Mn^{2+} , Ca^{2+} has been shown to affect the degradative activity of IDE *in vitro* and in intact cells (16, 17). As Ca^{2+} and other divalent cations have been implicated in some of the activities of insulin (18), the present study is directed at examination of IDE's binding, regulatory, and degradative functions, with special emphasis on the effect of metals.

Materials and Methods

[¹²⁵I]Iodinsulin specifically labeled on Tyr^{A14} was provided by Dr. Bruce Frank (Eli Lilly Research Laboratory, Indianapolis, IN) (19). Crystalline porcine insulin was provided by Dr. Ronald Chance (Eli Lilly Research Laboratory). Enzyme grade ammonium sulfate was purchased from ICN Biomedicals (Costa Mesa, CA). Succinyl-Leu Leu-Val-Tyr-7-amino-4-methylcoumarin (LLVY), Boc-Leu-Ser-Thr-Arg-7-amino-4-methylcoumarin (LSTR), and CBZ-Leu-Leu-Glu β -naphthylamide (LLE) were purchased from Sigma (St. Louis, MO). All chemicals were reagent grade or better. The enzyme preparation used was partially purified by ammonium sulfate fractionation (20). The enzyme was dialyzed overnight against at least 20 vol sodium acetate, pH 6.2, with no addition, 1 mM EDTA, or 1 mM EGTA, with three changes. EGTA is more specific for Ca^{2+} than EDTA, with EGTA binding Ca^{2+} about 2.5 times more tightly (21). Conversely, EDTA binds most other divalent cations (including Zn^{2+} , Mn^{2+} , and Co^{2+}) 10–10,000 times more tightly than EGTA. Acetate salts of the divalent cations were added at the concentrations indicated.

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Measurement of degradative activity

Insulin degradation was measured by trichloroacetic acid precipitation (22) and expressed as the percent soluble per 15 min. Degradations of LLVY, LSTR, and LLE were measured by incubating the enzyme sample with 13 μM fluorogenic peptide in 0.1 M Tris buffer, pH 7.5 (assay volume, 1 ml), for 60 min at 37 C on a metabolic shaker (12). Inhibitors or activators were included at the concentrations indicated. The reaction was stopped by the addition of 0.2 ml ethanol on ice. The increase in fluorescence was measured on a fluorometer with excitation and emission wavelengths of 390 and 440 nm for LLVY and LSTR, and 335 and 410 nm for LLE. Data are expressed as the change in fluorescence units per 60 min and normalized with respect to the activity of untreated, uninhibited enzyme.

Results

Insulin degradation by IDE is reduced by treatment with EDTA (Fig. 1). Ca^{2+} restores insulin degradative activity to EDTA-treated IDE at concentrations as low as 10^{-6} M. At high Ca^{2+} concentrations (10^{-3} M), activity is increased above that of untreated enzyme (Fig. 1).

The regulatory function of IDE is also affected by EDTA. Figure 2A shows that insulin inhibits LLVY degradation by the untreated IDE-MCP complex. The addition of Ca^{2+} slightly increases the activity of untreated complex, but does not alter the effect of insulin. EDTA treatment of the IDE-MCP complex decreases the chymotrypsin-like activity of MCP, as reflected by decreased degradation of LLVY (Fig. 2B). The addition of 10^{-5} – 10^{-3} M Ca^{2+} restores LLVY degradation, but not the insulin effect, although wide variability in the results can be seen.

There is evidence that IDE contains more than one divalent cation (15), a tightly bound Zn^{2+} and a more easily dissociable Mn^{2+} , and that the different cations may have different effects on IDE activities. Thus, the effect of a different chelator, EGTA, was examined (Fig. 2C). Again, LLVY degradation was decreased, with only a slight further effect of insulin. Ca^{2+} , however, restored both the LLVY degradative activity and the effect of insulin.

Similar studies with EDTA- or EGTA-treated IDE-MCP complex were performed with the readdition of other cations (Zn^{2+} , Mn^{2+} , and Co^{2+}) that alter IDE degradative activity

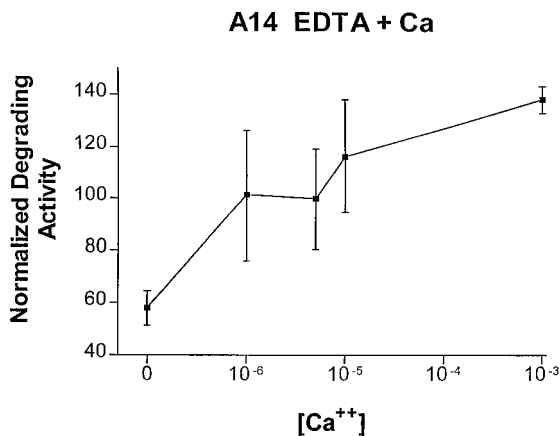


FIG. 1. Restoration by Ca^{2+} of EDTA inhibited insulin-degrading enzyme. EDTA treatment of IDE inhibits the enzyme by almost half, but readdition of Ca^{2+} restores the activity, and even increases it at 10^{-3} M. Values are the mean \pm SEM of IDE activity, expressed as a percentage of IDE without EDTA treatment.

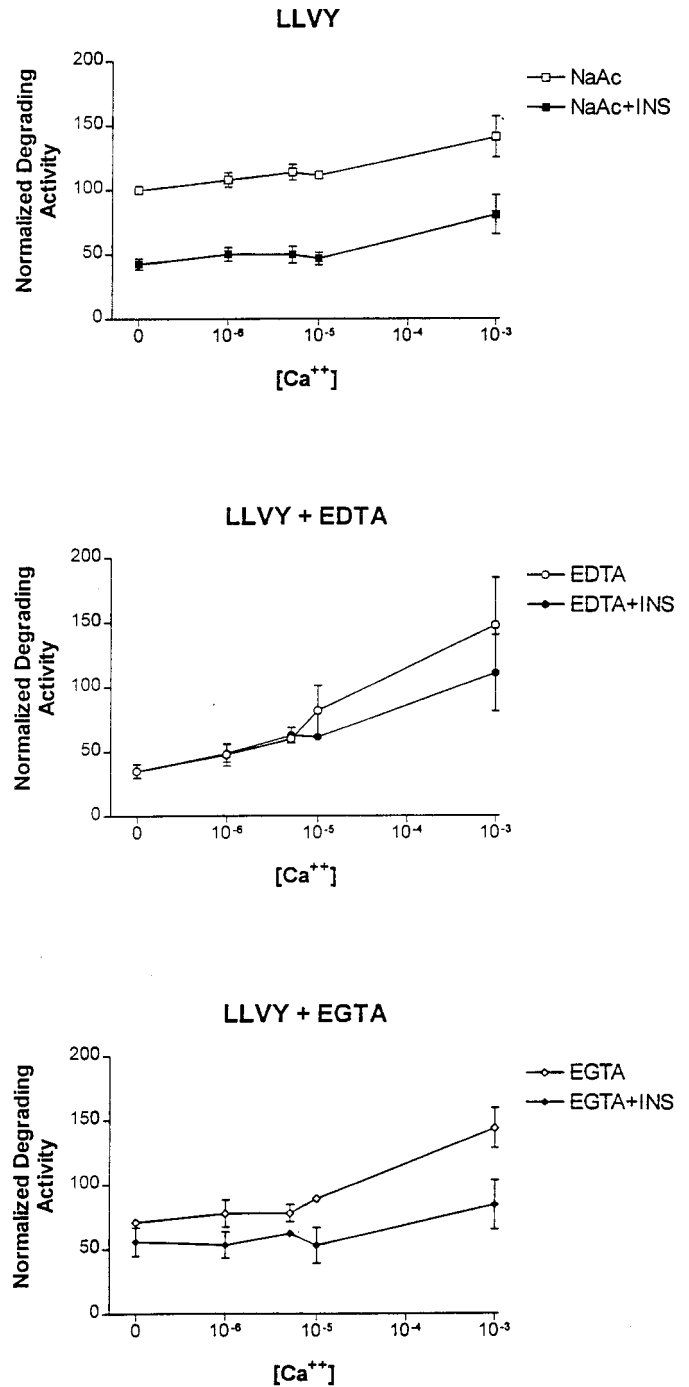


FIG. 2. Effect of Ca^{2+} readdition on MCP, EDTA-treated MCP, and EGTA-treated MCP. A, Addition of Ca^{2+} has a slight stimulatory effect on LLVY degradation, with no effect on insulin inhibition. B, EDTA treatment reduces LLVY activity by 60% and eliminates the response to insulin. Calcium addition reactivates the LLVY degradation, but does not restore insulin sensitivity. C, EGTA treatment reduces LLVY activity and essentially eliminates the response to insulin. Calcium addition restores both the degradative activity and the response to insulin. Values shown are the mean \pm SEM of LLVY activity, expressed as a percentage of that in the untreated enzyme.

(Figs. 3 and 4). The effects of Ca^{2+} are shown for comparison. The metals have variable effects on restoring MCP activity to the EDTA-treated complex, but none restores the insulin

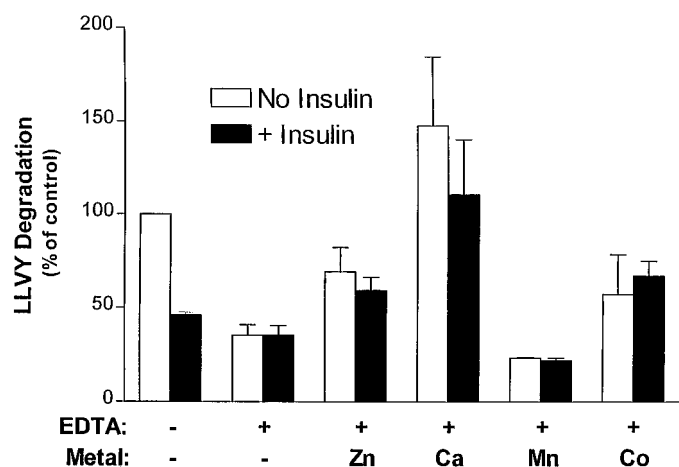


FIG. 3. The effect of EDTA treatment and metal readdition on LLVY-degrading activity and its inhibition by insulin. The graph shows the activity of the IDE-MCP complex toward LLVY after various treatments, without (*open bars*) and with (*closed bars*) the addition of $1 \mu\text{M}$ insulin. Partially purified IDE-MCP complex was dialyzed overnight against sodium acetate or sodium acetate with 1 mM EDTA, with readdition of various metals. Values shown are the mean \pm SEM of LLVY-degrading activity normalized to the activity of untreated enzyme with no insulin added. The metal chloride concentrations used were those that maximally restored IDE activity toward radiolabeled insulin and were as follows: Zn^{2+} , 10^{-6} M ; Ca^{2+} , 10^{-3} M ; Mn^{2+} , $5 \times 10^{-6} \text{ M}$; and Co^{2+} , 10^{-5} M .

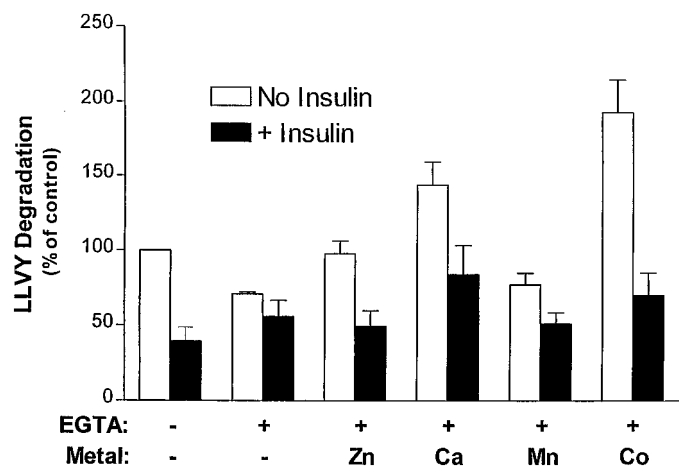


FIG. 4. The effect of EGTA treatment and metal readdition on LLVY-degrading activity and its inhibition by insulin. The graph shows the activity of the IDE-MCP complex toward LLVY after various treatments without (*open bars*) and with (*closed bars*) the addition of $1 \mu\text{M}$ insulin. Partially purified IDE-MCP complex was dialyzed overnight against sodium acetate or sodium acetate with 1 mM EGTA, with readdition of various metals. Values shown are the mean \pm SEM of LLVY-degrading activity normalized to the activity of untreated enzyme with no insulin added. As EGTA treatment had little effect on insulin-degrading activities, the metal concentrations used were the same as those used for the EDTA-treated enzyme: Zn^{2+} , 10^{-6} M ; Ca^{2+} , 10^{-3} M ; Mn^{2+} , $5 \times 10^{-6} \text{ M}$; and Co^{2+} , 10^{-5} M .

effect. However, Zn^{2+} , Ca^{2+} , and Co^{2+} restore the regulatory function of IDE on MCP activity in the EGTA-treated material. Manganese appeared to restore insulin sensitivity, but did not reach statistical significance ($P = 0.066$ in a one-tailed t test).

MCP has multiple catalytic sites. Insulin and IDE regulate

the trypsin-like (LSTR degradation) as well as the chymotrypsin-like (LLVY degradation) activity. EDTA treatment dramatically decreases LSTR degradation and eliminates the insulin effect (Fig. 5). The readdition of the divalent cations, Ca^{2+} and Co^{2+} , restores some degrading activity, but not the insulin effect. EGTA treatment reduces LSTR activity, but the change does not achieve significance and does not remove the insulin inhibition of that MCP activity (Fig. 6).

These data (Figs. 2–6) support a role for an easily dissociable divalent cation that is essential for the insulin effect on the complex (EGTA effects). EDTA treatment, however, results in more profound alterations, perhaps due to partial removal of a more tightly bound cation and subsequent conformational changes. Simple addition of individual cations did not restore functional control of the complex by insulin.

To further explore the regulatory role of IDE in control of MCP, selected inhibitors were examined (Table 1). The metalloproteinase inhibitor, 1,10-phenanthroline, inhibited IDE and abolished the insulin effect on LLVY degradation similar to EDTA. *N*-ethylmaleimide and bacitracin, known inhibitors of IDE, also blocked the effect of insulin. Phenylmethylsulfonyl fluoride, at low concentrations, had no appreciable effect.

Table 2 compares the effects of inhibitors of IDE on the proteolytic activity of the IDE-MCP complex and purified MCP. Phenanthroline, *N*-ethylmaleimide, and bacitracin inhibit LLVY degradation when MCP is complexed with IDE, but these agents are ineffective on purified MCP. These findings support a regulatory (activating) effect of IDE on MCP activity.

The importance of activation of MCP by IDE is supported by the effect of removal of IDE during purification. As reported previously (11, 12), IDE and MCP (LLVY degradation) copurify through various standard and affinity procedures, but can be separated on ion exchange columns. The IDE-MCP complex was purified through ammonium sulfate fractionation, diethylaminoethyl batch separation, phenyl-Sepharose, P200, and chromatofocusing. The preparation was then chromatographed on a Mono-Q HPLC column with all LLVY-degrading

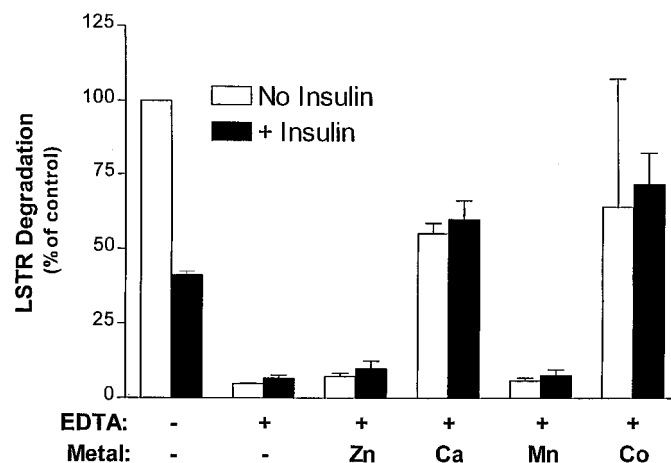


FIG. 5. The effects of EDTA treatment and metal readdition on LSTR-degrading activity and its inhibition by insulin. See Fig. 3 for details.

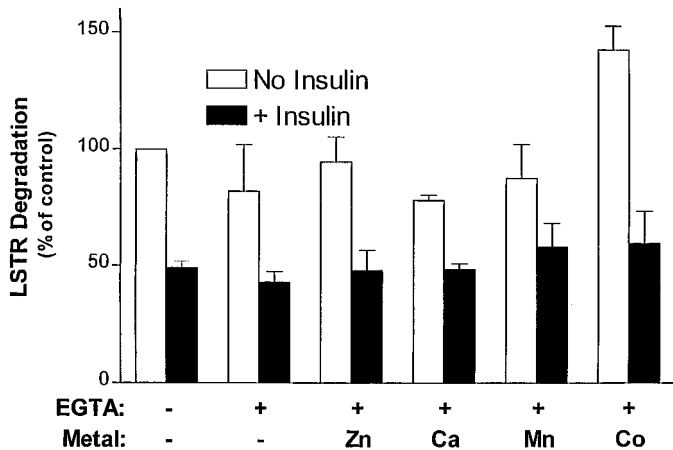


FIG. 6. The effects of EGTA treatment and metal readdition on LSTR-degrading activity and its inhibition by insulin. See Fig. 4 for details.

TABLE 1. The effects of various inhibitors on the IDE-MCP complex

Addition	Concentration	IDE (%)	LLVY degradation	
			-Insulin	+Insulin
None		100	100	51.3
1,10-Phenanthroline	0.1 mM	44.4	73.1	76.2
	1.0 mM	0.2	30.6	30.6
PMSF	0.1 mM	93.7	111.2	68.0
	1.0 mM	85.4	116.1	62.7
NEM	0.01 mM	56.0	63.4	66.0
	0.1 mM	13.4	24.7	18.6
Bacitracin	10 μ g/ml	59.9	71.3	52.0
	100 μ g/ml	24.5	39.2	35.4
PCMB	0.05 mM	5.1	^a	^a
	0.5 mM	6.4	^a	^a
EDTA	1.0 mM	52.4	33.7	28.8
	10 mM	7.9	32.8	30.1

The IDE-MCP preparation was prepared as in *Materials and Methods*. Insulin and LLVY degradation were measured in the presence of the inhibitors or vehicle at the indicated concentration. The degradation of LLVY was determined in the presence of 1.0 μ M insulin or vehicle only. Values are expressed as a percentage of the degrading activity in the presence of vehicle only. The data are the means of four independent experiments.

^a No LLVY degradation.

TABLE 2. The effects of inhibitors of IDE on MCP activity

Additions	LLVY degrading activity (% of control)	
	IDE-MCP	Purified MCP
None	100	100
Phenanthroline (0.2 mM)	21	95
NEM (0.2 mM)	3	100
Bacitracin (1.0 mM)	10	85

activity eluting in a sharp, symmetrical peak (not shown). The recovery of LLVY-degrading activity from the ion exchange column was 0.02%, and the specific activity decreased from 6.2 to 1.1 (fluorescent units per μ g protein/h).

IDE control of MCP activity may be due to a conformational change in MCP, as this enzyme is responsive to re-

agents that alter its conformation (23). SDS at low concentrations increases the activity, with higher concentrations inhibiting (24). To explore this, dose-response curves with SDS were performed on the IDE-MCP complex without and with insulin, and the effects on LLVY, LSTR, and LLE activities were examined (Fig. 7). LLVY-degrading activity was stimulated by SDS at concentrations between 0.015–0.030%. Insulin inhibited LLVY and LSTR, but not LLE, degradation in the absence of SDS, but had no effect or had even a slight stimulatory effect in the presence of SDS.

To determine if the insulin effect on the complex could be restored, IDE-MCP was activated by SDS, and then the SDS was removed (Table 3). Degradation of LLVY was monitored continuously, and insulin was added to achieve sequential concentrations of 10^{-9} , 10^{-8} , and 10^{-6} M. As can be seen, MCP activity was inhibited at all insulin concentrations after removal of SDS.

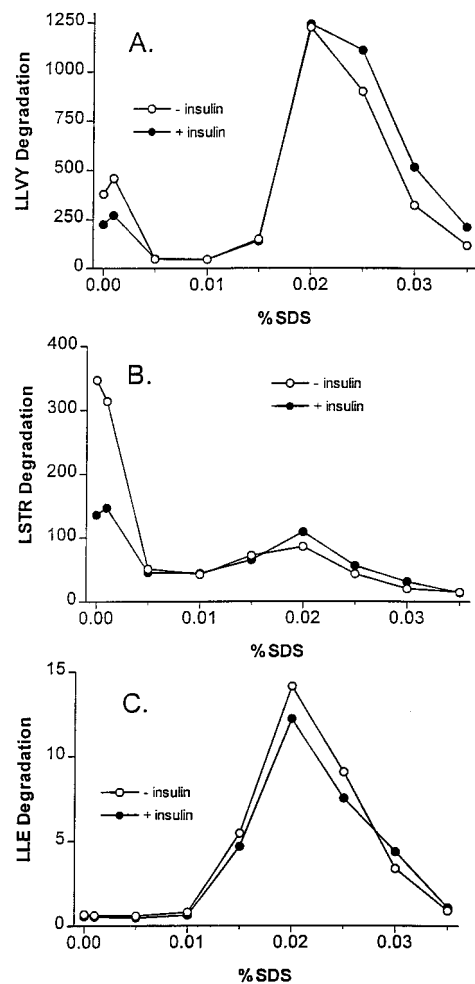


FIG. 7. Insulin has no effect on peptide degradation by SDS-activated multicatalytic proteinase. Enzyme (ammonium sulfate-purified complex) was incubated with increasing concentrations of SDS (0–0.035%, wt/vol) in the presence (●) and absence (○) of 1 μ M insulin. Degradation of peptides is expressed as fluorescence liberated (arbitrary units) per 60 min. LLE degradation is expressed as thousands.

TABLE 3. Effect of insulin on MCP after treatment and removal of SDS

Insulin (M)	LLVY degradation
0	14.1
10^{-9}	9.1
10^{-8}	7.9
10^{-6}	5.6

LLVY activity is given as the change in fluorescence units per minute. The IDE-MCP complex was treated with 0.02% SDS, and the sample was incubated with Bio-Beads SM2 (Bio-Rad) to remove excess SDS.

Discussion

Although a role for IDE in cellular insulin metabolism is firmly established (7), many factors mitigate against this being the sole physiological activity of the enzyme. Although all of insulin-sensitive tissues contain the enzyme, it is also found in noninsulin-sensitive tissues and in multiple subcellular compartments. IDE is found in peroxisomes (25–27), cytosol (7), and endosomes (28) and on plasma membranes (29). The cellular content of IDE is developmentally regulated, and IDE has been implicated in cellular differentiation (26, 30–32) and in growth factor binding (30). These diverse properties become less confusing if IDE is recognized as a multifunctional protein that can complex with a variety of subcellular components (proteasome, steroid receptor, and peroxisomes) and alter the activities of these complexes.

The stimulatory effects of IDE on proteasomes and the glucocorticoid and androgen receptors are established by the present and previous reports (9, 12), but the mechanism is not. The reversible nature of the activation argues against proteolytic activation of the complex by IDE. Other possibilities include conformational changes in the complex, IDE degradation of the ligand, proteolytic products generated by IDE, or some combination of these.

The present study provides evidence that divalent cations are involved in the activity and control of the IDE-MCP complex. Treatment of the partially purified enzyme with EDTA significantly reduced the activity of IDE, which can be restored by the addition of Ca^{2+} with as little as 10^{-6} M. This effect is presumably mediated by removal of the Zn^{2+} found at the active site of IDE, which is required for activity. This treatment also reduces the chymotrypsin-like and trypsin-like activities of the proteasome and eliminates the ability of insulin to inhibit these activities. The addition of Ca^{2+} or Co^{2+} restores the chymotrypsin-like and trypsin-like activities, but fails to restore the insulin responsiveness. Zn^{2+} and Mn^{2+} show relatively little effect.

We also examined the effect of EGTA on the complex. EGTA has a slightly higher affinity (2.5 times) for Ca^{2+} than does EDTA, but has 10–10,000 times less affinity for other cations (including Zn^{2+}) (21). Under the conditions of these experiments, EGTA had no effect on IDE activity (data not shown), but did reduce the chymotrypsin-like activity of the proteasome and effectively eliminated the insulin effect. A similar reduction was seen with trypsin-like activity, although it did not reach statistical significance. Insulin's ability to inhibit was restored by some of the cations (Ca^{2+} and Co^{2+}). These data suggest that there are two metals involved in the complex, as we have reported previously (15).

We propose that one metal, zinc, is tightly bound to IDE, and is essential for its activity, and the other, possibly Mn^{2+} or Ca^{2+} , is more loosely bound and is required for the productive interaction of IDE and the proteasome. EDTA can chelate the tightly bound Zn^{2+} and the more loosely bound metal associated with the complex. This inhibits IDE, thereby lessening its stimulatory effect on the proteasome. Loss of the Zn^{2+} molecule causes a conformational change in IDE, dissociating it from the proteasome and disrupting the ability of insulin to alter proteasome activity. Readdition of divalent cation restores IDE activity, but because it is no longer part of the complex, IDE cannot alter proteasome function. However, EGTA only has effects on the more loosely bound metal and disrupts IDE's ability to affect chymotrypsin-like activity, but does not cause IDE to dissociate from the complex. Thus, addition of cations can restore insulin sensitivity.

The fact that IDE must be active and in a complex with the proteasome to mediate its regulatory effects is further supported by the data presented in Tables 1 and 2. Agents that inhibit IDE lessen proteasome activity and effectively eliminate insulin's inhibitory effect.

Finally, we studied the IDE proteasome interactions using SDS, which has been shown to alter proteasome conformation. As shown in Fig. 7, the chymotrypsin and trypsin-like activities of the proteasome are insulin sensitive in the absence of SDS, but lose both sensitivity and considerable activity at 0.005% SDS. Presumably, this is due to SDS altering the interaction between IDE and the proteasome. At higher concentrations, SDS further alters the conformation of the proteasome and stimulates the chymotrypsin-like and peptidyl glutamyl-degrading activities. However, at these high concentrations, IDE is still unable to interact with the proteasomes, and insulin does not inhibit. This effect is reversible.

The data presented here characterize and further support the idea that IDE can serve as a regulator of the proteasome. Metal ions play important roles in this interaction. First, IDE is a metalloproteinase and must be active for insulin's inhibitory effect. Further, a second, more loosely bound divalent cation is required for IDE to mediate its effects. Changes in protein conformation are important, as illustrated by the loss of activity when IDE has its active site metal removed, and by changes in activity induced by SDS.

The physiological effect of insulin is to inhibit cellular protein degradation, with greater effects during catabolic states (e.g. starvation, diabetes, and traumatic stress). As cell survival requires continued protein turnover, it would not be expected that insulin could totally inhibit MCP activity or completely block protein degradation. Rather, the IDE-MCP interaction may serve as an activator of basal cellular protein degradation, analogous to activation of the glucocorticoid and androgen receptors by IDE (9), with insulin and other substrates then acting to inhibit the stimulated process. Thus, selective effects on cellular protein degradation in insulin-sensitive cells are under the control of insulin-IDE interactions. MCP comprises a relatively large proportion of the protein in many cells (up to 1%), and IDE, although expressed at high levels in some tissues (33), is not present in sufficient quantities in all tissues to be a universal regulator of all of MCP's activities. Our results show that insulin sub-

stantially inhibits only two of the activities of MCP, the chymotrypsin- and trypsin-like activities. The proteasome may have as many as five different proteolytic activities (34). It also remains to be determined whether the degradation of ubiquitinated proteins is inhibited by insulin through IDE.

Although not widely appreciated, many studies have shown biological activity of intracellular insulin (35, 36) and alterations in insulin activity with inhibition of cellular insulin processing (37, 38). Our recent studies have provided a potential mechanism for these previously unexplained observations. In this scheme, the IDE portion of the complex acts as an intracellular receptor for insulin and possibly other growth factors. The insulin-IDE interaction then alters activity of the protein complex (*i.e.* MCP, glucocorticoid receptor, androgen receptor, and perhaps others) and contributes to the biological actions of insulin. The insulin effect on MCP can be prevented by altering the activity of IDE. Although speculative, cellular activity regulated by the IDE complexes may be affected by cellular redox state and divalent cation distribution due to changes in IDE. Alterations in insulin action have been associated with ion flux and redistribution and with redox state. These effects could be explained in part by regulatory effects of IDE. Further studies are required.

Acknowledgment

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