ATP Inhibits Insulin-Degrading Enzyme Activity[1]

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We studied the ability of ATP to inhibit *in vitro* the degrading activity of insulin-degrading enzyme. The enzyme was purified from rat skeletal muscle by successive chromatographic steps. The last purification step showed two bands at 110 and 60 kDa in polyacrylamide gel. The enzyme was characterized by its insulin degradation activity, the substrate competition of unlabeled to labeled insulin, the profile of enzyme inhibitors, and the recognition by a specific antibody. One to 5 mM ATP induced a dose-dependent inhibition of insulin degradation (determined by trichloroacetic acid precipitation and insulin antibody binding). Inhibition by 3 mM adenosine 5′-diphosphate, adenosine 5′-monophosphate, guanosine 5′-triphosphate, pyrophosphate, β-γ-methyleneadenosine 5′-triphosphate, adenosine 5′-O-(3 thiotriphosphate), and dibutiryl cyclic adenosine 5′-monophosphate was 74%, 4%, 38%, 46%, 65%, 36%, and 0%, respectively, of that produced by 3 mM ATP. Kinetic analysis of ATP inhibition suggested an allosteric effect as the plot of 1/v (insulin degradation) versus ATP concentration was not linear and the Hill coefficient was more than 1 (1.51 and 2.44). The binding constant for allosteric inhibition was $K_i^T = 1.5 \times 10^{-7}$ M showing a decrease of enzyme affinity induced by ATP. We conclude that ATP has an inhibitory effect on the insulin degradation activity of the enzyme. [Exp Biol Med Vol. 226(4):334–341, 2001]

Key words: insulin degradation; ATP inhibition; insulin-degrading activity

Insulin-degrading enzyme (IDE) is an evolutionarily conserved protein. *In vitro,* IDE not only degrades insulin and glucagon, but also insulin growth factors I and II, the leader peptide of peroxysomal prethiolase (1) transforming growth factorα, the β-amyloid peptide (2), and other peptides (reviewed in Ref. 3). *In vivo,* IDE degrades insulin (4) and transforming growth factor α (5). Cleavage sites studies suggest that substrate recognition by IDE would depend on the tertiary peptide conformation more than on an amino acid sequence (2).

Degradation activity by IDE is abolished by the Zn^{2+} chelator 1,10-phenantroline, the insulin-binding inhibitor bacitracin, the thiol blocking agents p-hydroxy-mercuribenzoate, and N-ethylmaleimide among others (3, 6).

Since IDE is a protease able to remove insulin and growth factors, the enzyme and the mechanism that regulates its activity can play some role in the process of gene expression and cellular growth (7). Nevertheless, the physiological role of IDE is yet to be established.

Insulin degradation is conditioned by binding to its receptors and internalization, but the mechanisms of IDE regulation are still unknown. The peptides mentioned above could be competitive inhibitors of insulin degradation by IDE only if they reach the required concentrations in the cells. The reported kinetic values make it difficult to establish their role as competitive inhibitors (reviewed in Ref. 8). An insulinase inhibitor was proposed long ago (9) and later, a low-molecular weight inhibitor of IDE, which competes with insulin for binding, was found (10). The physiological role of this inhibitor still remains unclear.

It has been demonstrated that 5 mM extracellular ATP inhibits the insulin degradation in isolated rat adipocytes (11), suggesting that extracellular ATP may be a signal for inhibition in the degradative pathway of insulin processing. In addition, inhibition of insulin degradation was found in rat cytosol treated with aluminum fluoride or phosphatidylserine plus ATP (12).

In this paper we demonstrate that ATP inhibits the insulin degradation activity of IDE.

Materials and Methods

Materials. Male Wistar rats (150–200 g) were donated by Dr. P. Gullace from INAME (National Institute of Medicaments, Buenos Aires, Argentina). Porcine insulin was a gift of Dr. Anderson (Lab. Beta, Buenos Aires, Argentina), and somatotropin (hGH) was a gift from Bio-Sidus (Buenos Aires, Argentina). $Na^{125}I$ (17.4 Ci/mg specific activity) was obtained from DuPont (Boston, MA). DEAE-sephadex, superfine sephadex G-25, and sephadex G-50

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was obtained from Pharmacia (Uppsala, Sweden). Sephacryl S-200, pentyl agarose, PB-94 (polybuffer exchanger), polybuffer 74, ATP, ADP, AMP, GTP, β, γ-methyleneadenosine 5'-triphosphate (AMP.PCP), and adenosine 5'-O-(3-thiotriphosphate; ATPγS), dibutiryl cyclic AMP (DcAMP), dithiothreitol (DTT), N-ethylmaleimide (NEM), 1,10-phenantroline (OP), bacitracin, phenylmethylsulfonyl fluoride (PMSF), Coomassie Brilliant Blue R and Silver Stain kit were manufactured by Sigma Chemical (St. Louis, MO). Sodium pyrophosphate (PPi) was from Carlo Erba (Milano, Italy). Reagents for SDS-PAGE and immunoblotting were purchased from Bio-Rad (Richmond, CA). All drugs were of analytical grade.

Enzyme Purification. The purification procedure was similar to that described by Kishner et al. (13) and Shii et al. (14), with some changes. Two hundred grams of rat skeletal muscle were taken from pectoral muscle, dorsum, and legs, and was homogenized in sucrose 0.25 M (3 ml/g of muscle), centrifuged at 1000*g* for 20 min, and the supernatant was centrifuged again at 13,000*g* for 20 min. The supernatant was precipitated with 30% to 60% ammonium sulfate and when the total muscle extraction reached 1400*g*, all the precipitates were dissolved in 20 mM Tris-HCl, pH 7.4, and dialyzed three times: two times against 10 volumes of the same buffer during 4 hr and the last against 20 volumes of the same buffer during 15 hr. All the dialyzed material was applied by batch adsorption to DEAE-sephadex A-50 (29 g of protein in 350 ml of resin) also equilibrated with the above buffer. The resin was washed with 500 ml of the same buffer plus 150 mM NaCl, and the insulin-degrading activity was eluted with 500 ml of 250 mM NaCl in 20 mM Tris-HCl, pH 7.4, and was then precipitated again with 30% to 60% ammonium sulfate. The 30% to 60% fraction was dissolved in 20 mM Tris-HCl, pH 7.4, dialyzed as described, and subject to DEAE-sephadex A-50 chromatography (400-ml column). The resin was washed with 2 L of 20 mM Tris-HCl buffer, pH 7.4, 130 mM NaCl, and was eluted each time with 500 ml of 180 and 250 mM NaCl discontinuous salt gradient in the same buffer. The 250 mM NaCl fraction showed the highest degrading activity. Degradation inhibited by EDTA was selected, pooled, precipitated with ammonium sulfate (45%–58%), and applied to a 10-ml pentyl agarose hydrophobic column previously equilibrated with 45% ammonium sulfate in 10 mM imidazole-HCl buffer, pH 7.1. Each column was loaded with ~60 mg of proteins and three columns were required for purification. Each column was eluted with a discontinuous gradient of 28% and 23% ammonium sulfate in the same buffer and the elution was continued until proteins reached the baseline. Most of the degrading activity was present in the 23% gradient. Tubes with the higher activities were pooled, precipitated with 60% ammonium sulfate, and desalted by chromatography in superfine sephadex G-25 (50-ml column) equilibrated with 25 mM histidine-HCl buffer, pH 5.5. The peak of insulin-degrading activity was again pooled and concentrated on the Centriprep 30 (Amicon, Beverly, MA). This material was applied to a 10-ml chromatofocusing PB 94 column and was eluted with Polybuffer 74. The degrading activity was eluted between pH 5.2 and 4.8 and the peak was pooled and precipitated with 60% ammonium sulfate. The precipitate was dissolved in 20 mM Tris-HCl, pH 7.4, 10% glycerol and was applied for chromatography (1.5 ml with ~2 mg) to Sephacryl S-200 (100-ml column) equilibrated with the same buffer and eluted in 1-ml fractions.

To check the ATP inhibition with a more purified enzyme, IDE was purified from chromatofocusing with FPLC (Pharmacia (Uppsala, Sweden) in a Mono Q column equilibrated with 20 mM Tris-HCl buffer, pH 7.4, and eluted with a lineal NaCl gradient from 0 to 500 mM.

All the above steps were performed at 4°C. The purified enzyme was stored at −70°C until use. Protein concentration was assayed by absorbance at 280 nm in a spectrometer (Spectronic 3000, Milton Roy, Inc.) or using Lowry's or Bradford's methods. Samples of all purification steps were subjected to 7.5% SDS-PAGE and stained with Coomassie Blue or silver.

Degradation Assay. Insulin degradation was studied at 37°C for 15, 30, or 45 min (as indicated in each experiment) in 50 mM Tris-HCl buffer, pH 7.4, with 1% bovine serum albumin (BSA), 10 mM $MgCl_2$, 2 mM $MnCl_2$, and 16 to 20 pM of ^{125}I-insulin (final incubation volume of 100 μl). ^{125}I-porcine insulin was labeled with the Chloramine T method and was purified with preparative starch gel electrophoresis followed by superfine sephadex G-50 chromatography. Insulin was predominantly labeled in tyrosine A-14 with a specific activity between 250 and 300 mCi/mg and conserved biological activity (15). Conditions were selected to preserve a linear degradation during the incubation period. The reaction was stopped by the addition of TCA (5% final concentration), kept overnight at 4°C, and then centrifuged for 15 min at 1000*g*, and then the pellets and supernatants were separated. Degradation was calculated as the increase in ^{125}I-insulin fragments (TCA soluble). TCA precipitation underestimates insulin degradation, but it is widely used for its simplicity and reproducibility (16, 18). In some experiments insulin degradation was also assessed in the presence of antibody excess, and bound insulin was separated by dextran-charcoal.

All experiments were performed in duplicate or triplicate. ^{125}I-insulin controls (no enzyme present) had more than 92% of intact insulin; hence, experimental results were corrected for this value. Basal insulin degradation with IDE is shown in each experiment. Radioactivity was counted in a gamma counter (Packard Instrument Co., Downers Grove, IL).

Preparation of IDE Antibody. The synthetic peptide YKEMLAVDAPRRHK (p15) corresponding to residues 940 to 953 of rat insulin-degrading enzyme sequence (19) plus a cysteine added to N-terminal was prepared by Instituto de Quimica (Universidade de Sao Paulo, Brazil). The peptide was coupled to keyhole limpet hemocyanin

(KLH) using maleimide (imject-activated immunogen conjugation kit, Pierce, Rockford, IL), mixed with complete Freund's adjuvant and injected into two Norfolk-Californian female rabbits (LD or LM). The coupled peptide preparation was 175 μg of p15 per 1 mg of KLH.

The immunization scheme was as follows: 150 μg of coupled p15 was injected into rabbits in the first and the second injection (first booster). The second and the third boosters were 50 μg of coupled p15. All boosters were performed monthly using incomplete Freund's adjuvant. Blood was collected after 15 to 20 days of each booster, and the antibody was tested for specific reactivity with the labeled peptide (p15). Antibody titer was controlled by RIA (5 pg/ml of ^{125}I-p15) and variable serum dilution. When an adequate titer of anti-p15 was reached, specificity and sensitivity was controlled with cytosol from different rat tissues using a 1/30,000 dilution of anti-p15.

Anti-p15 was immunopurified by affinity chromatography using an agarose-bound peptide (p15) column (Sulfolink Coupling gel, Pierce). After elution with 0.1 mM glycine, pH 2.5, the eluate containing anti-p15 was dialyzed and concentrated. Both IgG antibody and immunopurified IgG antibody were used. Immunoblot comparison of LD antibody (450 μg/ml) suggests about 700 times purification of IgG anti-p15. Proteins were estimated with Bradford's method and BSA as standard.

Kinetic Studies. Preliminary experiments were done in order to determine the linearity of insulin degradation during the incubation period. K_m studies were determined with 2 to 4 μg of IDE, incubated at 37°C with 20 pM of ^{125}I-insulin for 30 min and increasing amounts of insulin (1 nM to 1 μM), in a final volume of 100 μl. The insulin degradation was determined as mentioned before and ATP inhibition was measured at three concentrations (0.3, 1, and 3 mM). Because insulin degradation described a Michaelis-Menten reaction, the apparent K_m was calculated taking the statistical variations into account. To ensure the linearity in the double reciprocal plot, we performed experiments measuring the insulin degradation rate versus ATP at 0, 0.3, 0.6, 1, and 3 mM ATP with insulin concentration at 10^{-8} M and 5×10^{-8} M.

Identical experimental conditions were used for all the kinetic studies, and three or four sets of separate experiments in duplicate or triplicate were done. Insulin and ATP were expressed at molar concentration, and the velocity of insulin degradation was calculated per hour.

We applied the Hill plot to further analyze the experimental results. The ATP inhibition was calculated as described by Levit et al: log $[v/v_i - 1]$ versus log inhibitor (20). The binding constant for an allosteric inhibitor was determined with an allosteric model (21).

Statistical comparisons between groups were performed using Student's t test.

Results

Purification and Characterization of IDE. Table I shows the purification steps of IDE from rat muscle homogenates. We used, with poor results, the specific antibody p15 to obtain immunopurified IDE by affinity chromatography using an anti-p15-sepharose column. This antibody recognizes a sequence on the C-terminal region of the enzyme that is only present in rIDE and hIDE or in its degradation products containing this sequence. Self-proteolysis of its C-terminal region by native enzyme or antibody saturation by degraded IDE carrying its C-terminal region could explain this result.

The purification degree of IDE in each step was estimated by insulin degradation and immunoblotting with the specific antibody p15 (not shown). The Sephacryl S-200 purification step does not reach complete purification, as can be seen in Figure 1. Silver staining of SDS-PAGE of Sephacryl S-200 shows five bands (220, 180, 110, 60, and 50 kDa; Fig. 1A). An additional purification step with gradient chromatography in Mono Q with FPLC shows a band at 110 kDa, the reported molecular weight of IDE, and another at 60 kDa, which could correspond to the amino-terminal portion of the enzyme (Fig. 1B). This last band persists during the different purification steps; after Mono Q, it is proportionally increased in relation to IDE.

Degraded IDE is present in many steps of IDE purification. We can not confirm that split IDE really have or do not have insulin degradation activity because we have not isolated and purified degraded IDE. Split IDE apparently do not have activity. We only observed insulin degradation when the 110-kDa band was present, and degradation was proportional to the amount of this band. The Mono Q pu-

Table I. Purification Steps of Insulin-Degrading Enzyme

	Volume (ml)	Proteins (mg/ml)	Total proteins (mg)	Degradation (pM/min)	Specific activity (pM/min/mg)
Homogenate	3116	8.949	27,885.08	0.06939	2.65×10^{-6}
DEAE (batch)	505	28.7145	14,500.82	0.01531	1.06×10^{-5}
DEAE	70	5.33341	373.34	0.09664	3.52×10^{-4}
Pentyl agarose	18.3	10.346	189.33	0.05755	7.15×10^{-3}
PB94-chromatofocusing	7	1.3322	9.33	0.09819	0.01313
Sephacryl S200	11	0.01923	0.21	0.05051	0.2387

Note. ^{125}I-Insulin: 8 pM; final volume: 100 μl; incubation time: 15 min at 37°C. The degradation was linear during the incubation time. Protein was assayed with Lowry's method and the protein standard curve was adjusted to protein concentration.

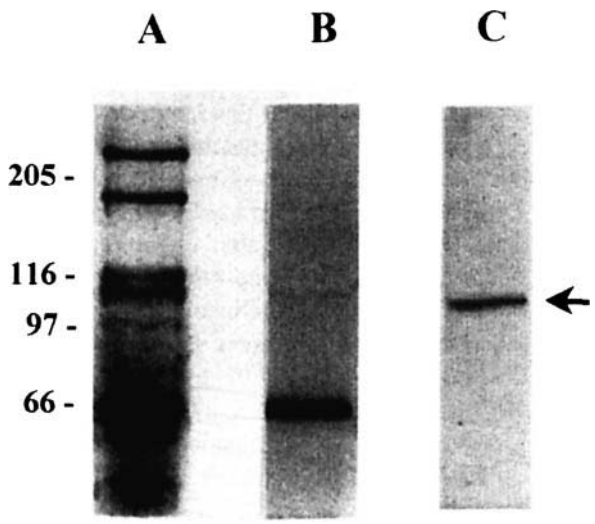

Figure 1. SDS-gel electrophoresis of rIDE purification steps. Boiled samples were electrophoresed at 7.5% acrylamide gel. Ten micrograms of protein were applied on each lane. (A) Silver-stained gel after Sephacryl S200. There are five bands at 220, 180, 110, 60, and 50 kDa. (B) Coomassie Blue-stained gel after Mono Q purification of IDE. There are two bands at 110 kDa (the reported M_r of IDE) and another at 60 kDa. (C) Immunoblotting of IDE with the specific antiserum p15 (immunopurified IgG) in the step of Sephacryl purification.

rification had a 7.92% of insulin degradation activity. This activity was proportional to IDE band and did not agree with the 60-kDa band that appeared as split IDE.

Due to the instability of highly purified IDE, the following studies were performed with the enzyme purified up to the Sephacryl S-200 step unless otherwise indicated.

In order to further characterize IDE, the performance ability of various agents that prevent insulin degradation were investigated (Table II). The sulfhydryl-modifying reagent NEM is the most potent inhibitor of IDE. The metal chelate 1,10-phenantroline decreases insulin degradation even though no pre-incubation had been performed, and bacitracin, an IDE inhibitor that competes with insulin, also inhibits protease activity. IDE insulin degradation is not modified by the serine protease inhibitor PMSF.

NEM, OP, and bacitracin are well-known inhibitors of insulin-degrading enzyme, referred to as thiol-dependent metalloprotease. Enzyme specificity was tested by the addition of excess unlabeled insulin (10 μg/tube), which completely inhibited the degradation of labeled insulin, whereas hGH, a nonrelated peptide, had no effect on insulin degradation. The degradation activity found agreed with the wide band of IDE shown in Sephacryl S-200 and also observed in the immunoblot with the specific IDE antibody p15 (Fig. 1C).

As all these results suggest, we have purified IDE and the studies really refer to the enzyme.

Table II. IDE Inhibition by Different Agents

Agent	Concentration	Insulin degradation (% inhibition)	*n*
NEM	1 mM	91.47 ± 1.67	3
1,10-Phenantroline	1 mM	40.08 ± 8.00	3
Bacitracin	1 mg/ml	86.52 ± 2.27	3
Insulin	10 μg/tube	91.56 ± 0.53	3
hGH	10 μg/tube	7.87 ± 7.87	3
PMSF	1 mM	8.87 ± 6.70	3

Note. Basal insulin degradation was 16.48 ± 1.89 in three sets of separated experiments and this value was considered 100%. IDE: 2 to 4 μg. Incubation time: 45 min. Data represent mean ± SE. *n* = number of experiments.

Interaction of ATP and Other Nucleotides with IDE. Previous experimental studies showed the absence of direct interactions between Zn^{2+} and ATP (not shown) and the same conclusion can be reached from this study.

Addition of 10 mM $MgCl_2$ to the incubation media increases insulin degradation by 22.4% ± 0.66% (n = 3). Zn^{2+} chelation denatures IDE, which lost its activity, and Zn^{2+} and Mn^{2+} ions protect IDE from denaturation. Mn^{2+} and Mg^{2+} ions are required for ATP inhibition, and the effect of 3 mM ATP on insulin degradation is half in the absence of these ions in the incubation buffer (Table III). Consequently, direct Zn^{2+} interaction with ATP is unlikely because in such a case, Mn^{2+} and Mg^{2+} should be unnecessary; they are required for ATP inhibition.

ATP with both ions added to the incubation media inhibits insulin degradation in a dose-dependent fashion (Fig. 2A). The inhibition of insulin degradation at 5 mM ATP is almost complete.

The following experiments were performed to determine if the effect of ATP was direct or due to the presence

Table III. IDE Inhibition of NaF and Nucleotides ITS Comparison with ATP

Nucleotide (3 mM)	Percent of inhibition	*n*
ATP 3 mM (control)	100	
ATP (no Mn^{2+} or Mg^{2+})	53.65 ± 4.31	3
ADP	74.03 ± 4.18	6
AMP	4.46 ± 0.81	3
PPi	46.23 ± 3.24	6
GTP	38.39 ± 8.21	3
AMP.PCP	65.33 ± 2.15	3
ATPγS	36.19 ± 7.87	3
NaF (50 mM)	0.0	3
ATP + NaF (50 mM)	84.73 ± 3.00	3
DcAMP	0.0	3
DcAMP (0.1 mM)	0.0	3
ATP + DcAMP (0.1 mM)	96.61 ± 3.16	3
GTP (0.1 mM)	0.0	3
ATP + GTP (0.1 mM)	97.55 ± 4.07	3

Note. Inhibition with 3 mM ATP was considered 100%. Insulin degradation with IDE in three sets of separated experiments with different enzyme batchs was: basal IDE, 16.20 ± 1.89%, plus 3 mM ATP: 5.84 ± 1.75% (n = 6); basal IDE, 13.86% ± 0.82%, plus 3 mM ATP: 1.98% ± 0.49% (n = 3); basal IDE, 9.87% ± 0.54%, plus 3 mM ATP, 0.43% ± 0.11% (n = 3). IDE concentration in each assay: 2 to 4 μg. Incubation time: 45 min. Data represent mean ± SE. n = number of experiments in duplicate or triplicate in each series.

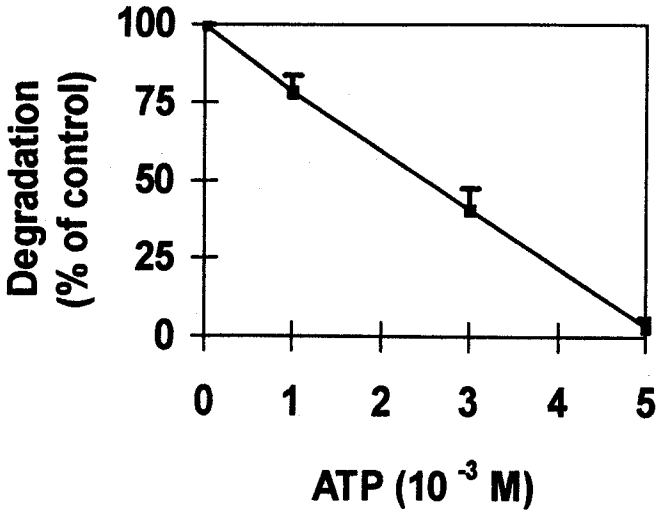

Figure 2. ATP inhibition of insulin degradation. Two to 4 μg of purified IDE were incubated during 45 min at 37°C with 20 pM ^{125}I-insulin in the presence of increasing concentrations of ATP. Basal insulin degradation without ATP (18.41% ± 2.69%) is the mean ± SE of at least six set of separate experiments in duplicate.

of other proteins because Sephacryl S-200 purification of IDE showed other bands besides the enzyme (Fig. 1). Both NaF, a broad phosphatase inhibitor, and at 50 mM an activator of G proteins, and GTP, an activator of G proteins at micromolar concentrations, had no inhibitory effect. GTP had some inhibitory effect, but only at high (millimolar) concentrations (Table III). Three millimolar DcAMP had no effect on IDE insulin degradation, suggesting that cyclic AMP, in our study conditions, does not stimulate a contaminant protein kinase, does not increase ATP effect, and does not compete with ATP on IDE. These results would confirm absence of a contaminant protein that could result in inhibition of IDE insulin degradation.

The inhibitory effects of ADP, AMP, GTP, PPi, and two nonhydrolyzable ATP analogues, AMP.PCP and ATPγS, were studied. All nucleotides induce inhibition of insulin degradation, but there is a quantitative difference between them (Table III). The behavior of nucleotides and nonhydrolyzable ATP analogues may suggest phosphate requirement for total IDE inhibition, but they are not sufficient evidence for a definite conclusion. The results only suggest that the inhibition of degradation is probably a consequence of ATP-IDE interaction because ATP is the most active and specific nucleotide for this inhibitory response.

To verify these experimental results we studied insulin degradation with 5% TCA and antibody excess simultaneously (Table IV). The percent of insulin degradation with both methods gave similar results, although the TCA method underestimates the absolute value.

Finally, IDE purified by chromatography in Mono Q and incubated with 5 mM ATP gave essentially the same result (IDE, basal degradation: 7.92 ± 0.06; 5 mM ATP, 0.44 ± 0.04; percentage of inhibition: 97.09 ± 4.13; media ± SE; n = 3). This makes the participation of proteins other than IDE in the effect of ATP unlikely.

Kinetic Studies. Initially, we studied the influence of ATP on enzyme kinetics. The double reciprocal plot of insulin degradation by IDE gives a straight line with an apparent K_m of 58×10^{-9} M (Fig. 3A). Insulin degradation by IDE shows a decrease in V_{max} with increasing doses of ATP (V_{max}, M/hr; IDE = 1.32×10^{-8}; IDE + 0.3 mM ATP = 0.95×10^{-8}; IDE + 3 mM ATP = 0.49×10^{-8}), and the Lineweaver-Burk plot with ATP also gives straight lines. There is a significant decrease in the V_{max} of insulin degradation between control and IDE plus 3 mM ATP ($P < 0.05$) with a similar K_m (IDE + 0.3 mM ATP, K_i: 56×10^{-9} M; IDE + 3 mM ATP, K_i: 45×10^{-9} M; see inset). These results initially suggested a noncompetitive inhibition.

A cooperative interaction might give this response, depending on the range of concentrations tested and the purification degree. We studied from 0.3 to 3 mM ATP (one order of magnitude), but it is difficult to measure a wider ATP range due to the inhibition of insulin degradation. To demonstrate that linearity was true we performed experiments of insulin degradation at two insulin concentrations and variable ATP doses.

The plot of 1/v (insulin degradation) versus ATP (Dixon plot) is not linear and suggests a cooperative effect of ATP on IDE (Fig. 3B). To confirm these results we analyzed our experimental data using the Hill plot. Hill coefficient of insulin degradation versus insulin substrate

Table IV. Insulin Degradation with Antibody Excess and TCA

	Antibody excess			TCA 5%	
	Insulin degradation	Percent value		Insulin degradation	Percent value
Experiment A					
IDE (n = 3)	29.38 ± 1.93	100	(n = 3)	9.87 ± 0.54	100
IDE + 3 mM ATP (n = 3)	0.51 ± 0.51	1.78 ± 1.02	(n = 3)	0.34 ± 0.18	3.75 ± 2.03
Experiment B					
IDE (n = 2)	34.43 ± 4.07	100	(n = 3)	13.86 ± 0.82	100
IDE + 3 mM PCP.ATP (n = 2)	15.78 ± 4.83	44.81 ± 8.74	(n = 3)	6.10 ± 0.42	43.92 ± 0.78

Note. Insulin degradation was measured with 5% TCA or insulin antibody excess. Insulin antibody (IAS 105) was used for the assay in a 1/20,000 dilution. The separation technique was dextran-charcoal and the incubation time was 3 days. The basal insulin degradation was considered 100% in each method. Data represent mean ± SE. n = number of experiments in duplicate.

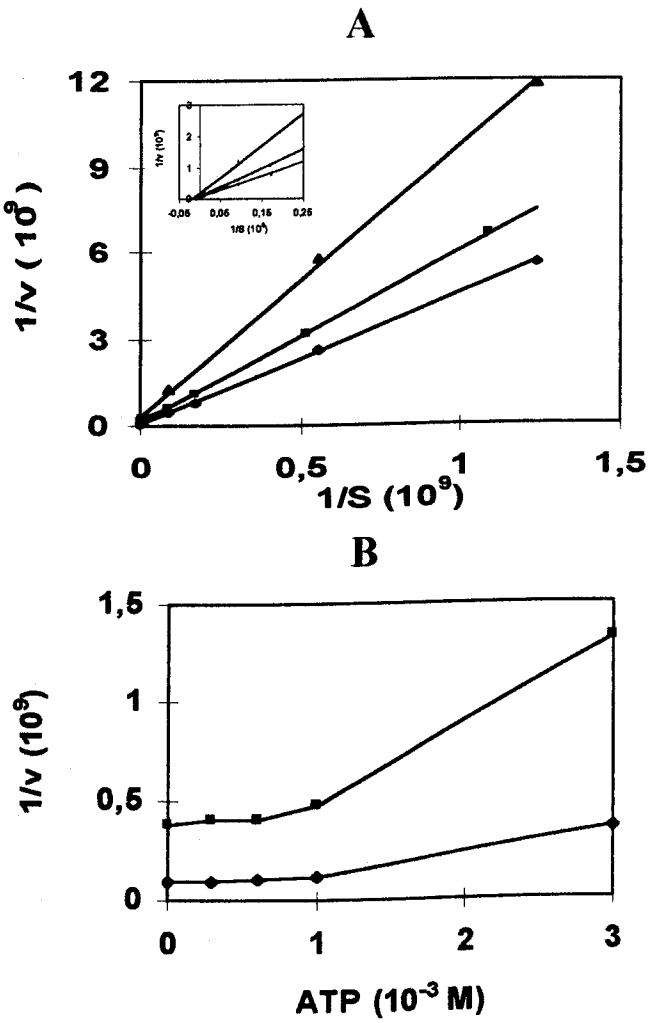

Figure 3. (A) Lineweaver-Burk plot of IDE inhibition by ATP. Increased amount of native insulin (1 nM to 1 μM) and 0.3 or 3 mM ATP were used for K_m and K_i studies. IDE (▲) K_m: 58×10^{-9} M; IDE + 0.3 mM ATP (■) K_i: 56×10^{-9} M; IDE + 3 mM ATP (♦) K_i: 45×10^{-9} M. The lines were generated by linear regression ($r^2 = 0.99$). Basal insulin degradation was IDE: 11.06% ± 2.02%; IDE + 0.3 mM ATP, 8.25% ± 1.20%; IDE + 3 mM ATP, 5.21% ± 1.81%. Increasing doses of ATP decrease the V_{max} of insulin degradation (M/h). IDE: 1.32×10^{-8}; IDE + 0.3 mM ATP: 0.95×10^{-8}; IDE + 3 mM ATP: 0.49×10^{-8}. Results are the mean ± SE of four sets of separate experiments in duplicate. Inset, The X interception in the double reciprocal plot is shown in another scale. (B) 1/v (insulin degradation) versus ATP. Two to 4 μg of purified IDE was incubated during 30 min at 37°C with 20 pM ^{125}I-insulin and two concentrations of unlabeled insulin: 10^{-8} M (■) or 5×10^{-8} M (♦). For this study 0.3, 0.6, 1, and 3 mM ATP were used. The plot is not linear at both insulin concentrations, suggesting allosteric interactions between IDE and ATP. Results are the mean ± SE of four experiments in duplicate.

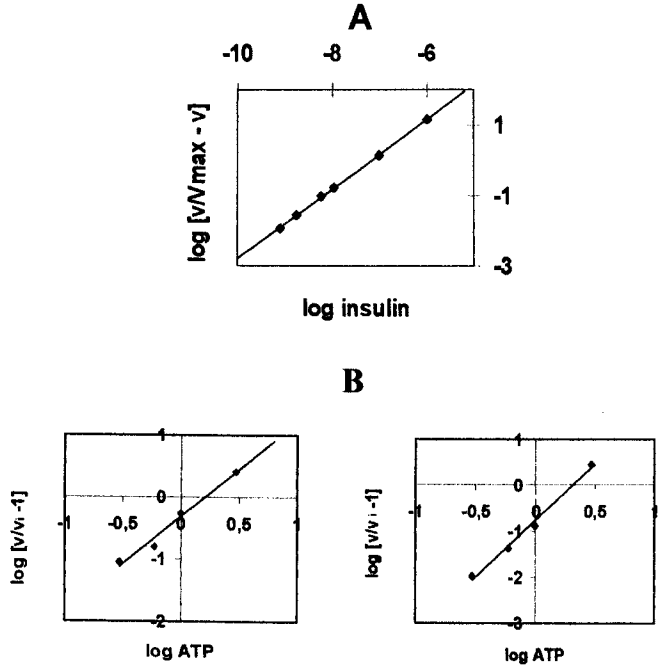

Figure 4. A- Hill plot of insulin degradation versus insulin substrate. The Hill coefficient is 0.99 ($r^2 = 0.99$). The line was calculated by linear regression. Insulin degradation follows a Michaelis-Menten kinetic. Experimental conditions are the same as in Figure 2. (B) Hill plot of insulin degradation versus ATP. Values for the Hill plots were taken from Figure 3B and calculated at two insulin concentrations. The Hill coefficient is 1.51 ($r^2 = 0.97$, 10^{-8} M insulin, ■) and 2.44 ($r^2 = 0.99$, 5×10^{-8} M insulin, ♦). Both values suggest an allosteric interaction between IDE and ATP. The lines were calculated by linear regression.

was 0.990 ($r^2 = 0.99$; Fig. 4A), but Hill coefficient of insulin degradation versus ATP was higher than 1 (Hill plot of 10^{-8} M insulin: 1.51 and 5×10^{-8} M insulin: 2.44). Direct calculation from Dixon plot gave essentially the same result (10^{-8} M insulin: 1.57 and 5×10^{-8} M insulin: 2.83). Other plots such as v/V versus log s at variable ATP doses gave sigmoid curves, showing again an allosteric interaction (not shown).

Calculation of insulin degradation binding constant with ATP as allosteric inhibitor, with the assumption of two IDE interacting sites for ATP or two functional associated units of IDE, gave a $K_i^T = 1.5 \times 10^{-7}$ M, which represents a decrease of IDE affinity for insulin.

Discussion

The expression of IDE is regulated in different tissues and also during development and growth, as shown in *Drosophila* and rats (7, 22), but regulation of IDE activity is not known. Akiyama et al. (10) observed a dissociation between IDE concentration and IDE degrading activity, which was ascribed to an IDE inhibitor. Regulation by ATP of IDE activity in isolated cells was not found in an early work performed at 22°C (23), yet a regulation of IDE by extracellular ATP was proposed in a work with adipocytes at 37°C (11). In this way P2 purinoreceptors cascade is probably followed by some protein phosphorylation and inhibition of IDE activity. In free cytosol, inhibition of IDE degrading activity following addition of Al_3F or phosphatidylserine plus ATP was attributed to protein phosphorylation after activation of a G-protein and protein kinase C. Protein-protein interaction was suggested for this inhibitory response (12).

We found almost complete inhibition of insulin degradation with the addition of 5 mM ATP to purified IDE. The addition of Mg^{2+} to IDE increases degradation as observed

by us and others (4, 9, 24). Native IDE only has Zn^{2+} and trace amounts of Mn^{2+} in its composition as previously reported (3). Nevertheless, the ATP inhibitory response requires divalent cations like Mn^{2+} and Mg^{2+} essential for ATP binding to proteins.

Inhibition of insulin degradation appears as a consequence of specific ATP activity because the other nucleotides do not reach the same ATP inhibition of insulin degradation. Contaminant proteins do not participate in this effect. Inhibition of degradation (for example, through phosphorylation) by contaminant proteins should be observed with the addition of GTP, DcAMP, or NaF (Table III) because 3 mM ATP does not suppress insulin degradation, and interaction with a contaminant protein could overcome (more than 100%) the ATP inhibition. The results suggest that there is no interaction with other proteins in the ATP inhibitory effect.

If we look at the two nonhydrolyzable ATP analogs and compare with the inhibition induced by PPi, we might suggest that PPi could be required for the inhibitory effect on IDE. The AMP inhibitory effect apparently agrees with this observation. Thus, the available evidence suggests that the inhibition of degradation is probably a consequence of ATP-IDE interaction. ATP is the most active and specific nucleotide for this inhibitory response, and it also inhibits insulin degradation at the same concentration that is found in cells.

This ATP inhibition of degradation does not preclude other regulatory mechanisms of insulin degradation as protein-protein interactions or phosphorylation (11, 12). Many proteins are ATP regulated. For example, in bovine adrenal chromaffin cells ATP activates K(Ca) channels in a dose-dependent manner and might even regulate catecholamine release by changing the cell membrane potential (25). Molecular chaperones participate in protein degradation and are dependent on ATP hydrolysis for activation (reviewed in Ref. 26).

Kinetic results suggest that ATP is a modifier of IDE. A Lineweaver-Burk plot of inhibition of insulin degradation at two ATP doses was linear, while in allosteric inhibition, deviation from linearity should be expected. As stated by Koshland (27), in a purified enzyme it is unlikely to find a number of polymorphic forms and electrostatic repulsion that can lead to large deviations. The Hill coefficient and other plots suggest that ATP is an allosteric inhibitor of IDE, and the Hill value found would suggest that more than one ATP interacts with IDE, or two units of IDE interact with ATP.

IDE elutes at an apparent M_r of 170 or 300 kDa (14, 28, 29), depending on the chromatographic system used, but in reduced conditions, its M_r is around 110 kDa (14). Furthermore, as published, cytosol cross-linking followed by IDE-specific immunoprecipitation shows a band of 160 kDa in SDS-PAGE formed by 110-kDa IDE and probably a 50-kDa due to split enzyme (30). IDE has also been found as a complex formed by 110-kDa IDE and a new 70-kDa cytosolic protein, which might regulate IDE transport between cytosol and peroxisomes (31). In addition, it has been proposed that in native conditions, IDE is a dimer (29). This information suggests that IDE could be a dimer or an interacting protein complex in native conditions. In our study the amino terminal portion of IDE remaining during IDE purification (60-kDa protein) might participate in the ATP inhibitory response.

Inhibition of degradation starts at micromolar concentrations of ATP that produce an inhibitory effect on IDE with a decrease of insulin affinity, as suggested by the allosteric K_i^T determination. If we suppose that this *in vitro* behavior of the enzyme would be similar in cells, ATP appears as the most important inhibitor. At cellular concentrations, ADP would have an inhibitory response of 7% compared with ATP because the relationship of ATP/ADP, critical for ATP synthesis, is close to 10/1. ATP concentration is almost constant in cells and only changes in organelles like mitochondria. The direct effect of other nucleotides like GTP, AMP, or cyclic AMP present in cells at lower concentrations would be less active or not active at all.

We observed that ATP also inhibits other IDE substrates like IGFI and amiloyd β-peptide. ATP could probably inhibit the degradation of other hormones like glucagon because it is known that insulin and glucagon are degraded by IDE (16). Nevertheless, cellular processing of insulin and glucagon are different. Insulin degradation is an intracellular event, and glucagon degradation is almost all extracellular (32, 33). Studies of endocytotic vesicles formation with insulin and glucagon showed that glucagon internalization is scarce (17, 34) and an extracellular protease probably accounts for the extracellular degradation of glucagon (32, 35, 36). The IDE cell behavior in hormone degradation and its inhibition by ATP probably depend on the type of cellular processing, suggesting that degradation by IDE in cells and *in vitro* might differ.

It is widely accepted that insulin degradation is started by IDE (2, 4). Our results indicate that ATP inhibits the insulin degradation by IDE. It is also known that the enzyme is found in the cytosol, the same as insulin after its internalization (3). At cytosolic ATP concentrations, the enzyme would be partially inhibited. This agrees with the kinetic of inhibition of insulin degradation and does not contradict other type of propositions regarding insulin degradation control (11, 12).

These *in vitro* findings might suggest that in cells, ATP levels could regulate intracellular insulin concentration depending on cell metabolic status; that is, cellular levels of ATP that depend on insulin for substrates availability would participate in the regulation of intracellular insulin through the modulation of its degradation. In this line we might suggest that insulin degradation is a controlled and regulated process. The experimental results shown in this work suggest that IDE-insulin interaction is probably more complex than previously assumed.

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