

ATP Effects on Insulin-degrading Enzyme Are Mediated Primarily through Its Triphosphate Moiety*

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Eun Suk Song‡, Maria Aparecida Juliano§, Luiz Juliano§, Michael G. Fried‡, Steven L. Wagner¶, and Louis B. Hersh‡||

From the ‡Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, Kentucky 40536-0084, the §Department of Biophysics, Escola Paulista de Medicina, São Paulo 04023-900, Brazil, and ¶Neurogenetics Inc., La Jolla, California 92037

It has been reported previously that ATP inhibits the insulin reaction (Camberos, M. C., Perez, A. A., Udrisar, D. P., Wanderley, M. I., and Cresto, J. C. (2001) *Exp. Biol. Med.* 226, 334–341). We report here that with 2-aminobenzoyl-GGFLRKHGQ-ethylenediamine-2,4-dinitrophenyl as substrate, ATP and other nucleotides increase the rate >20-fold in Tris buffer. There is no specificity with respect to the nucleotide; however, ATP is more effective than ADP, which is more effective than AMP. Triphosphate itself was as effective as ATP, indicating it is this moiety that is responsible for activation. The binding of triphosphate was shown to be at a site distinct from the active site, thus acting as a noncompetitive activator. With the physiological substrates insulin and amyloid β peptide, nucleotides and triphosphate were without effect. However, with small physiological peptides such as bradykinin and dynorphin B-9, ATP and triphosphate increased the rate of hydrolysis ~10-fold. Triphosphate and ATP shifted the oligomeric state of the enzyme from primarily dimer-tetramers to a monomer. These data suggest the presence of an allosteric regulatory site on insulysin that may shift its specificity toward small peptide substrates.

Insulysin (insulin-degrading enzyme (IDE),¹ EC 3.4.24.56) in conjunction with neprilysin, endothelin-converting enzyme, plasmin, and possibly other peptidases provides a major catabolic pathway for amyloid β peptides in the brain (1–3). A decrease in the activity of one or more of these peptidases would lead to increased A β and its subsequent oligomerization and deposition. The most convincing evidence that IDE participates in A β clearance is the finding of elevated levels of both A β_{1-40} and A β_{1-42} in the brains of mice in which the IDE gene

was disrupted (4, 5). Recent genetic evidence suggests, but has yet to be firmly established, that IDE is linked to late onset Alzheimer's disease (6–8). Any such linkage between IDE and late onset Alzheimer's disease is likely through the ability of IDE to degrade amyloid β peptides.

We recently reported that IDE displays the characteristics of an allosteric enzyme, whereby peptide substrates increase the rate of hydrolysis of other peptide substrates 2–7-fold (9). This behavior could be attributed to two effects as follows: a peptide-dependent shift in the monomer-oligomer equilibrium of IDE to favor a dimer, and a conformational change produced by the binding of peptide to one subunit that increased the activity of the adjacent subunit.

The finding that IDE displays allosteric properties led us to investigate further the reported effect of ATP and other nucleotides acting as noncompetitive inhibitors of the IDE-dependent hydrolysis of insulin (10). The results of this study show that nucleotide triphosphates can increase the rate of cleavage of small peptide substrates of IDE, and that the effect of nucleotide triphosphates can be attributed primarily to the triphosphate moiety. Triphosphates bind to a site distinct from the substrate-binding site. Thus IDE appears to contain an allosteric-like regulatory site that is distinct from the active site.

MATERIALS AND METHODS

Synthesis of the fluorogenic substrates Abz-GGFLRKHGQ-EDDnp, Abz-GGFLRKMGG-EDDnp, and Abz-GGFLRKAGQ-EDDnp were as described previously (11). Recombinant rat IDE was produced using the baculovirus system with expression of the enzyme in Sf-9 cells as a fusion protein containing an N-terminal hexahistidine joined through a linker region containing a tobacco etch virus (TEV) protease site (9). Recombinant His₆-IDE was purified to homogeneity by chromatography on a His-Select HC nickel affinity gel column (Sigma). The hexahistidine affinity tag as well as the linker region was removed by treatment with TEV protease in 20 mM potassium phosphate buffer, pH 7.3. Removal of the hexahistidine was confirmed by Western blot analysis using an anti-hexahistidine antisera obtained from Clontech.

Dynorphins A-7, A-8, A-9, A-10, A-13, and A-17 and dynorphins B-9 and B-13 as well as insulin were obtained from Bachem. β -Endorphin was obtained from Multiple Peptide Systems through the National Institute on Drug Abuse Research Tools program. Tyrosine A¹⁴-labeled ¹²⁵I-insulin and Tyr¹⁰-labeled ¹²⁵I-A β_{1-40} were obtained from Amersham Biosciences. Nucleotides were obtained from Sigma.

Activity Assays—The hydrolysis of the fluorogenic peptides was followed on a SpectraMax Gemini XS fluorescence plate reader using an excitation wavelength of 318 nm and an emission wavelength of 419 nm. Cleavage of these peptides between residues Arg and Lys produces an increase in fluorescence due to the separation of the fluorescent 2-aminobenzoyl (Abz) group from the fluorescent quenching ethylenediamine-2,4-dinitrophenyl (EDDnp) group (11). A standard curve was constructed by measuring the total fluorescent change produced by excess IDE or by trypsin at several different substrate concentrations. Because we initially used IDE as a fusion protein containing an N-

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|| To whom correspondence should be addressed: Dept. of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, 800 Rose St., Lexington, KY 40536-0084. Tel.: 859-323-5549; Fax: 859-323-1727; E-mail: lhersh@uky.edu.

¹ The abbreviations used are: IDE, insulin-degrading enzyme; HPLC, high performance liquid chromatography; Abz, 2-aminobenzoyl, EDDnp, ethylenediamine-2,4-dinitrophenyl, TEV, tobacco etch virus, PPP, triphosphate.

terminal hexahistidine joined to IDE through a polylinker, we determined whether there was any effect of the polylinker and hexahistidine on the reaction rate or on the effect of nucleotides. The polylinker and hexahistidine were removed by cleavage at a TEV protease site followed by separating IDE from TEV protease by gel filtration. There was no significant qualitative effect of the polylinker and hexahistidine on either the rate of the reaction or effects of nucleotides.

Insulin hydrolysis was followed by measuring the release of trichloroacetic acid-soluble radioactivity from ^{125}I -insulin labeled on tyrosine 14 of the A chain. Reaction mixtures (50 μl) contained 5 ng of recombinant IDE, 50 nM ^{125}I -insulin (specific activity $2 \times 10^5 \text{ cpm/nmol}$), 0.15% bovine serum albumin, and 50 mM Tris buffer, pH 7.4, as described previously (9). By applying electrospray ionization-mass spectrometry to reaction mixtures, we found that insulin was initially cleaved between Leu¹³-Tyr¹⁴ and Tyr¹⁴-Gln¹⁵ on the A chain and between Ser⁹-His¹⁰ and His¹⁰-Leu¹¹ on the B chain. The reaction rate was proportional to IDE up to 25% insulin cleavage.

The hydrolysis of Tyr¹⁰-labeled ^{125}I -A β_{1-40} was determined in 50- μl reaction mixtures containing 50 mM Tris buffer, pH 7.4, 65 nM ^{125}I -A β_{1-40} (specific activity $2 \times 10^5 \text{ cpm/nmol}$), and 10 ng of IDE. The reaction was initiated by the addition of IDE and was allowed to proceed for 60 min at 37 °C after which time the reaction was terminated by the addition of an equal volume of 15% trichloroacetic acid. After allowing the sample to sit on ice for 15 min, it was centrifuged for 5 min at 2,000 $\times g$ to separate the unreacted A β_{1-40} (pellet) from reaction products that remained in the supernatant. An aliquot of the supernatant was counted on a gamma counter. The cleavage of A β_{1-40} occurred initially at the His¹⁴-Gln¹⁵ and Phe¹⁹-Phe²⁰ sites (1) with the reaction being conducted under conditions in which 10% or less of the peptide was cleaved. Under these conditions the reaction rate was proportional to IDE.

Insulin hydrolysis was also followed by HPLC. Reaction mixtures containing 50 mM Tris buffer, pH 7.3, 10 μM insulin, 0.5 ng of IDE, and 2 mM ATP or 2 mM PPP_i when added were incubated for 15 min at 37 °C. The reaction was stopped by the addition of trifluoroacetic acid to 0.1% and then subjected to HPLC separation on a Vydac C₄ column employing a linear gradient of 5% acetonitrile in 0.1% aqueous trifluoroacetic acid to 50% acetonitrile in 0.1% aqueous trifluoroacetic acid. Peptide peaks were monitored by absorbance at 214 nm. Under these conditions of a high insulin concentration, the rate was proportional to IDE up to ~30% insulin cleavage. The hydrolysis of dynorphin-related peptides was followed in a similar manner by measuring the disappearance of the substrate peak.

Sedimentation Equilibrium Analysis—The effects of phosphate, ATP, and triphosphate (PPP_i) on the oligomeric state of IDE were determined by analytical ultracentrifugation at 4.0 ± 0.1 °C in a Beckman XL-A centrifuge using an AN 60 Ti rotor. The approach to equilibrium was followed by scanning the cell at 280 nm with a step size of 0.001 cm. Attainment of equilibrium was considered to be complete when scans made 6 h apart were indistinguishable. This was typically obtained in 20 h. Five scans were averaged for each sample at each rotor speed. Previous analyses carried out in phosphate buffer indicated that IDE self-associated in a monomer-dimer-tetramer pattern (9). Samples analyzed in 50 mM Tris buffer, pH 7.4, had a similar monomer-dimer-n-mer association pattern. In the presence of triphosphate or ATP, the highest multimer was significantly larger than a tetramer. Accordingly, sedimentation equilibrium data were analyzed according to Equation 1,

$$A(r) = \alpha_{m,0} \exp(\sigma_m(r^2 - r_0^2)) + \alpha_{d,0} \exp(2\sigma_m(r^2 - r_0^2)) + \alpha_{n,0} \exp(n\sigma_m(r^2 - r_0^2)) + \xi \quad (\text{Eq. 1})$$

where $A(r)$ is the absorbance at radial position r , and $\alpha_{m,0}$, $\alpha_{d,0}$, and $\alpha_{n,0}$ are the absorbances of monomer, dimer, and n -mer, respectively, at the reference radius (r_0). The parameter σ_m is the reduced molecular weight of the monomer ($\sigma_m = M_m(1 - \bar{v}/\rho)\omega^2/(2RT)$); M_m is the monomer molecular weight; \bar{v} is its partial specific volume; ρ is the solvent density; ω is the rotor angular velocity; R is the gas constant, and T is the absolute temperature. The base-line offset term ζ compensates for slight position-independent differences in the optical properties of different cell assemblies. Solvent density was measured using a Mettler density meter. The partial specific volume of IDE was calculated from its amino acid composition, using the method of Cohn and Edsall (12). Global analysis of data obtained at different rotor speeds was performed as first described by Johnson *et al.* (13) using the program NONLIN.²

² NONLIN for the Macintosh was obtained from www.cauma.edu software.

TABLE I
Effect of Mg^{2+} , Mn^{2+} , and ATP on Abz-GGFLRKHGQ-EDDnp hydrolysis by insulysin

Reactions contained 0.5 μg of IDE and 2 μM Abz-GGFLRKHGQ-EDDnp. The reaction rate in potassium phosphate buffer (0.32 $\mu\text{mol}/\text{min}/\text{mg}$) is 4.6 times faster than the reaction rate in Tris buffer. Values are the average of 2–4 determinations with different enzyme preparations given along with their standard errors.

Added reaction component	Relative rate with Abz GGFLRKHGQ-EDDnp	
	20 mM potassium phosphate, pH 7.3	50 mM Tris, pH 7.4
None	1.00	1.00
10 mM MgCl ₂	0.92 ± 0.02	1.28 ± 0.10
2 mM MnCl ₂	0.76 ± 0.04	0.97 ± 0.07
10 mM MgCl ₂ + 2 mM MnCl ₂	0.62 ± 0.03	1.13 ± 0.06
2 mM ATP	4.74 ± 0.28 ^a	24.9 ± 0.20 ^a
10 mM MgCl ₂ + 2 mM ATP	1.12 ± 0.04	1.83 ± 0.01 ^b
2 mM MnCl ₂ + 2 mM ATP	2.74 ± 0.64 ^a	3.82 ± 0.36 ^a
10 mM MgCl ₂ + 2 mM MnCl ₂ + 2 mM ATP	0.70 ± 0.17	19.6 ± 0.01 ^a

^a Data are relative to the “no addition” control, $p < 0.001$.

^b Data are relative to the no addition control, $p < 0.01$.

RESULTS

Effect of ATP and Other Nucleotides on the Hydrolysis of the Fluorogenic Substrate Abz-GGFLRKHGQ-EDDnp—Camberos *et al.* (10) reported that ATP, in the presence of Mn²⁺ and Mg²⁺, inhibited the IDE reaction as measured with ^{125}I -insulin as substrate. We attempted to confirm this finding by using a synthetic fluorogenic peptide substrate for IDE, Abz-GGFLRKHGQ-EDDnp. Cleavage of this substrate at the R-K peptide bond results in an increase in fluorescence due to relief of quenching of the N-terminal fluorescent Abz group by the C-terminal EDDnp group. Thus utilization of this substrate permits the continuous monitoring of the reaction and as such is more amenable to kinetic analysis.

The effect of ATP on the IDE-dependent cleavage of Abz-GGFLRKHGQ-EDDnp was measured by using the same metal ion and ATP concentrations as used in the study by Camberos *et al.* (10). As shown in Table I, we observed no significant effect of metal ions on the reaction. On the other hand, free ATP increased the rate in both phosphate and Tris buffer. In the presence of either Mg²⁺ or Mn²⁺, the activation by ATP was significantly reduced ($p > 0.001$), suggesting that the observed increase in reaction rate is due to free ATP and not a metal-ATP complex. It is also worth noting that the reaction in phosphate buffer (0.32 $\mu\text{mol}/\text{min}/\text{mg}$) is more than four times faster than in Tris buffer (0.07 $\mu\text{mol}/\text{min}/\text{mg}$). We found that the addition of 20 mM phosphate to a Tris-buffered reaction increased the rate 4.4-fold, essentially the same difference seen between phosphate buffer and Tris buffer. On the other hand adding increasing concentrations of Tris buffer to a constant concentration of phosphate buffer (20 mM) had no effect. Thus with the fluorogenic peptide as substrate, free phosphate produced activation of the IDE reaction, rather than inhibition as observed by Camberos *et al.* (10). Although the extent of activation was greater in Tris buffer, where the reaction rate was increased ~25-fold by 2 mM ATP, the absolute rates in the presence of 2 mM ATP were essentially the same in phosphate buffer (1.5 $\mu\text{mol}/\text{min}/\text{mg}$ IDE) as in Tris buffer (1.7 $\mu\text{mol}/\text{min}/\text{mg}$ IDE).

We next examined the nucleotide specificity for activation of the IDE reaction. As shown in Table II and in agreement with the results of Camberos *et al.* (10), ATP was more effective than ADP ($p < 0.001$), which was more effective than AMP ($p < 0.001$). However, there was no discernible specificity with regard to the particular nucleotide triphosphate, and triphosphate itself was as effective as a nucleotide triphosphate. An

TABLE II

The nucleotide specificity for activation of the IDE-dependent hydrolysis of Abz-GGFLRKHGQ-EDDnp

Reactions were conducted in 50 mM Tris buffer, pH 7.4, containing 0.5 µg of IDE, 2 µM Abz-GGFLRKHGQ-EDDnp, and 4 mM nucleotide mono-, di-, or triphosphate. Values given represent the average with the S.E. of two separate experiments with different enzyme preparations.

Nucleotide at 4 mM	Relative rate (IDE)
None	1.00
ATP	3.12 ± 0.03 ^a
ADP	2.20 ± 0.04 ^a
AMP	1.30 ± 0.01
dATP	3.72 ± 0.09 ^a
cAMP	0.80 ± 0.01
GTP	3.73 ± 0.03 ^a
GDP	2.52 ± 0.21 ^a
CTP	2.50 ± 0.19 ^a
TTP	2.67 ± 0.06 ^a
PP _i	1.45 ± 0.05 ^b
PPP _i	3.08 ± 0.06 ^a

^a Data are relative to the no addition control, $p < 0.001$.

^b Data are relative to the no addition control, $p < 0.05$.

estimate of the concentration of ATP or PPP_i needed to obtain 50% maximal activation (K_A) was determined from a graph of $1/v - v_0$ versus $1/[activator]$ (14), where v is the rate obtained in the presence of a given concentration of activator, and v_0 is the rate obtained in the absence of activator. A K_A of 1.4 mM was obtained for ATP, whereas a K_A of 1.1 mM was obtained for PPP_i. ATP maximally increased the reaction rate of 5 mM Abz-GGFLRKHGQ-EDDnp 38-fold, whereas PPP_i produced an 18-fold maximal increase in the reaction rate. These findings indicate that the adenosine moiety may make a small contribution to the activation effect seen with the fluorogenic substrate; however, activation seems to be primarily due to the negatively charged triphosphate moiety. This is in agreement with the data in Table I that show that the addition of a metal ion to ATP, which would shield the charge of the triphosphate group, decreased the extent of activation.

We looked at the ability of ATP and triphosphate to stimulate the hydrolysis of other fluorescent peptides. We found that ATP and PPP_i exhibited a dose-dependent increase in the rate of hydrolysis of Abz-GGFLRKHGQ-EDDnp with maximal increases of 16- and 10-fold, respectively. With Abz-GGFLRKAGQ-EDDnp as substrate, the maximal stimulation was ~25- and 12-fold by ATP and PPP_i, respectively. The K_A values for ATP were 0.7 and 1 µM for Abz-GGFLRKHGQ-EDDnp and Abz-GGFLRKAGQ-EDDnp, respectively, whereas for PPP_i K_A values of 0.6 and 0.8 µM were obtained. These are essentially the same K_A values noted above with Abz-GGFLRKHGQ-EDDnp as substrate.

Nucleotides Bind at a Site Distinct from the Active Site—We have shown previously that peptide substrates can increase IDE activity by binding at the active site of one subunit of the dimeric or tetrameric form of the enzyme and influencing the activity of the adjacent subunit (9). For example, bradykinin increases the rate of the IDE-dependent cleavage of Abz-GGFLRKHGQ-EDDnp some 4–6-fold. In order to determine whether ATP or triphosphate was acting at the same site as peptide substrates, we compared the ability of bradykinin to increase IDE activity in the presence of either excess ATP or excess triphosphate with the ability of bradykinin to increase IDE activity in the absence of ATP or triphosphate. As shown in Fig. 1, bradykinin increased IDE activity to nearly the same extent whether or not ATP or triphosphate was present. This suggests that ATP and triphosphate bind at a site distinct from the active site where bradykinin binds.

Nucleotides Affect the Rate of Substrate Hydrolysis—We next determined the effect of ATP and triphosphate on the kinetics

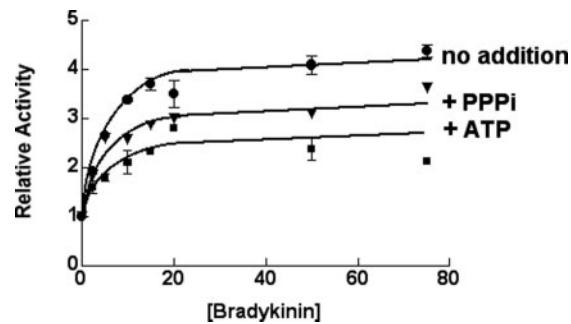


FIG. 1. The effect of bradykinin on the ATP-stimulated hydrolysis of Abz-GGFLRKHGQ-EDDnp. The rate of hydrolysis of 5 µM Abz-GGFLRKHGQ-EDDnp was measured in 50 mM Tris-HCl, pH 7.4, as a function of the indicated concentration of bradykinin in the presence or absence of ATP or PPP_i. The rate of substrate hydrolysis in the absence of added bradykinin, ATP, or PPP_i is 0.46 µmol/min/mg. In the presence of 4 mM ATP, but in the absence of bradykinin, this rate increased to 9.7 µmol/min/mg, whereas in the presence of 4 mM PPP_i, but in the absence of bradykinin, this rate was 3.5 µmol/min/mg.

of Abz-GGFLRKHGQ-EDDnp hydrolysis. Because the IDE reaction with Abz-GGFLRKHGQ-EDDnp as substrate displays sigmoidal kinetics, we fit the data to the Hill equation. As shown in Fig. 2, both ATP and triphosphate increase V_{max} . The value of V_{max} increases from 120 ± 4.6 µmol/min/mg in the absence of any addition to $1,714 \pm 76.6$ µmol/min/mg in the presence of 4 mM ATP and $1,115 \pm 36.8$ µmol/min/mg in the presence of 4 mM triphosphate. There was little, if any, effect on the $K_{0.5}$ with these values being 17.4 ± 1.1 µM in the absence of any addition, 11 ± 0.8 µM in the presence of ATP, and 11 ± 0.6 µM in the presence triphosphate. Similarly, the Hill coefficient was not significantly affected, being 2.2 ± 0.2 in the absence of any addition and 1.9 ± 0.2 and 2.4 ± 0.2 in the presence of ATP and triphosphate, respectively.

Effect of ATP and Other Nucleotides on the Hydrolysis of Physiological Peptides—Because the report of an inhibitory effect of ATP on the IDE reaction was determined with insulin as substrate (10), we next determined whether the activation we observed with the fluorogenic substrate and the reported inhibition with insulin were substrate-dependent. That this is the case is shown in Table III, where it can be seen that MnCl₂ and MgCl₂ either alone or in combination were without effect. Similarly, ATP either alone or in combination with metal ions had a rather small effect. We examined the effect of other nucleotides with insulin and another physiological peptide, amyloid β peptide 1–40, as substrates. With the possible exception of CTP with amyloid β peptide 1–40 as substrate, no significant effect was observed (Table IV).

We determined whether triphosphate or ATP caused a change in the substrate specificity of the enzyme by measuring insulin hydrolysis in phosphate buffer by HPLC in the presence and absence of 5 mM ATP or 5 mM PPP_i. The cleavage of insulin by IDE in the presence and absence of ATP or PPP_i produced the same product peaks, and the relative amounts of the cleavage products were unchanged. Thus ATP and PPP_i changed neither the rate of cleavage, the cleavage sites, nor the relative preference for a given cleavage site.

We next considered whether the size of the substrate would affect its ability to be stimulated by ATP. When the nonapeptide bradykinin was tested as a substrate at 20 µM in 50 mM Tris buffer, pH 7.4, ATP at 4 mM stimulated the rate of hydrolysis ~12-fold, whereas triphosphate at 4 mM stimulated the rate 10-fold. Similarly when the nonapeptide dynorphin B-9 was assayed at 20 µM, ATP (4 mM) and triphosphate (4 mM) stimulated the rate 8- and 11-fold, respectively. We thus tested a series of dynorphin A substrates of varying size for their ability to be affected by ATP and PPP_i. As seen in Table V,

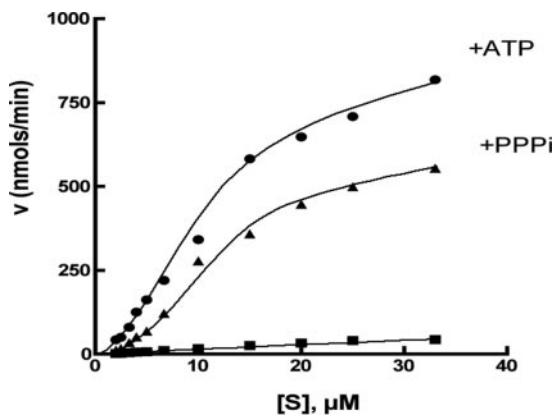


FIG. 2. The effect of ATP and PPP_i on the kinetics of Abz-GGFLRKHGQ-EDDnp hydrolysis. The hydrolysis of Abz-GGFLRKHGQ-EDDnp was measured in 50 mM Tris-HCl, pH 7.4, in the presence of 4 mM ATP, 4 mM PPP_i , or no addition. Data is normalized to 0.5 μg of enzyme.

TABLE III
Effect of Mg^{2+} , Mn^{2+} , and ATP on insulin hydrolysis by IDE

The IDE-dependent cleavage of insulin was determined as described under "Materials and Methods" using 50 nM ^{125}I -insulin as substrate in 50 mM Tris, pH 7.4. The rate of insulin hydrolysis in the absence of any addition is 2.3 $\mu\text{mol}/\text{min}/\text{mg}$. Data represent the average of two experiments with different enzyme preparations.

Added component	Relative rate of insulin hydrolysis
None	1.00
10 mM MgCl_2	0.88 ± 0.02
2 mM MnCl_2	0.86 ± 0.04
10 mM MgCl_2 + 2 mM MnCl_2	1.12 ± 0.14
2 mM ATP	0.84 ± 0.05
10 mM MgCl_2 + 2 mM ATP	0.63 ± 0.02^a
2 mM MnCl_2 + 2 mM ATP	0.65 ± 0.03^a
10 mM MgCl_2 + 2 mM MnCl_2 + 2 mM ATP	0.66 ± 0.06^a

^a Data are relative to the no addition control, $p < 0.01$.

TABLE IV
Effect of nucleotides on the IDE-dependent hydrolysis of insulin and $\text{A}\beta_{1-40}$

The IDE-dependent cleavage of insulin and $\text{A}\beta_{1-40}$ was determined as described under "Materials and Methods" in 50 mM Tris-HCl, pH 7.4, using 50 nM ^{125}I -insulin or 65 nM ^{125}I - $\text{A}\beta_{1-40}$ as substrate. The rate of $\text{A}\beta_{1-40}$ hydrolysis in the absence of any addition is 2.5 $\mu\text{mol}/\text{min}/\text{mg}$.

Nucleotide at 4 mM	Relative rate of insulin hydrolysis	Relative rate of $\text{A}\beta_{1-40}$ hydrolysis
None	1.00	1.00
ATP	1.14 ± 0.07	0.87 ± 0.01
ADP	1.05 ± 0.23	0.90 ± 0.04
AMP	1.26 ± 0.07	0.86 ± 0.05
dATP	0.83 ± 0.02	0.93 ± 0.06
cAMP	1.21 ± 0.13	0.89 ± 0.06
GTP	1.03 ± 0.02	0.82 ± 0.06
GDP	1.39 ± 0.02	0.86 ± 0.04
CTP	1.07 ± 0.12	0.64 ± 0.06^a
TPP	0.87 ± 0.14	0.80 ± 0.05
PP _i	0.75 ± 0.04	0.85 ± 0.08
PPP _i	0.70 ± 0.04 (0.59)	0.82 ± 0.03

^a Data are relative to the no addition control, $p < 0.05$.

stimulation of peptide hydrolysis by ATP and triphosphate seemed to be related to both peptide size and sequence. The hydrolysis of the smallest peptide tested dynorphin A-7 was only slightly affected by ATP or triphosphate, whereas within the dynorphin A series an ~3–5-fold stimulation was seen by ATP and an ~4–6-fold stimulation by triphosphate was seen for the octapeptide to the 13-mer. When the peptide size increased to 17 residues, no stimulation was observed. The re-

TABLE V
Effect of ATP and triphosphate on the IDE-dependent hydrolysis of dynorphin-related peptides of varying size

The IDE-dependent cleavage of the 10 μM dynorphin-related peptides were determined by HPLC by following substrate disappearance in 50 mM Tris-HCl, pH 7.4. Dynorphin A-17 is Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln; and dynorphin B-13 is Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr. The values in parentheses represent the fold increase in rate over the control reaction in Tris buffer.

Dynorphin peptide	Rate of hydrolysis $\mu\text{mol}/\text{min}/\text{mg}$	Rate of hydrolysis + 4 mM ATP $\mu\text{mol}/\text{min}/\text{mg}$	Rate of hydrolysis + 4 mM PPP _i $\mu\text{mol}/\text{min}/\text{mg}$
Dynorphin A-7	5.6	8.8 (1.6)	8.5 (1.5)
Dynorphin A-8	49.7	147 (3.0)	222 (4.5)
Dynorphin A-9	20	90 (4.5)	117.7 (5.9)
Dynorphin A-10	42.7	143 (3.3)	256 (6.0)
Dynorphin A-13	453	1190 (2.6)	1057 (2.3)
Dynorphin A-17	2413.3	2293.3 (1.0)	560 (1.1)
Dynorphin B-9	20	580 (29)	590 (29.5)
Dynorphin B-13	146	1006.7 (6.9)	1413.3 (9.7)

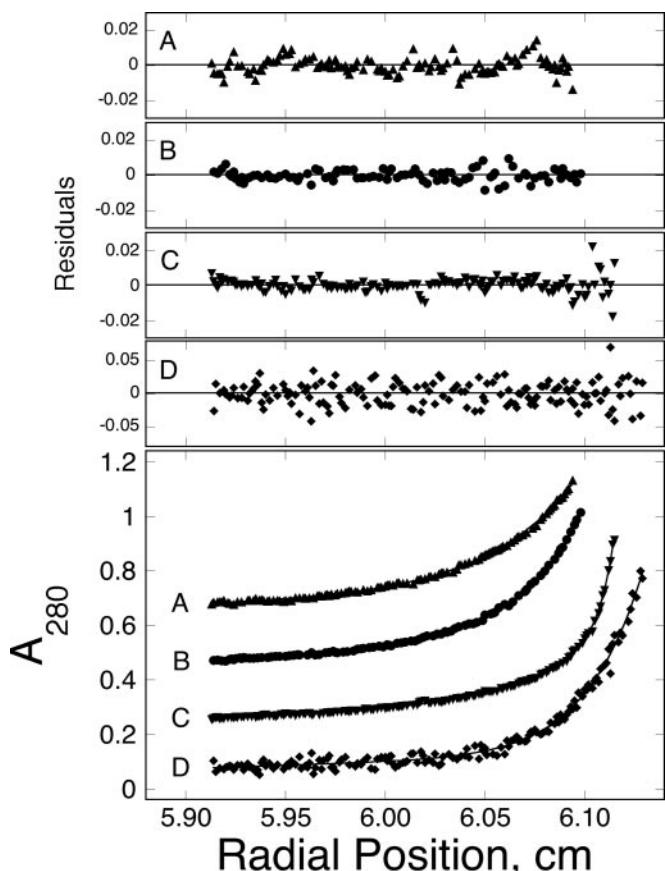


FIG. 3. Affect of ATP and PPP_i on the oligomeric state of IDE. Sedimentation equilibrium analysis of IDE in Tris buffer, phosphate buffer, and in the presence of triphosphate and ATP. Samples of IDE were dialyzed against 50 mM Tris (pH 7.4 at $20 \pm 1^\circ\text{C}$), curve A; 100 mM potassium phosphate (pH 7.3 at $20 \pm 1^\circ\text{C}$), curve B; 50 mM Tris (pH 7.4 at $20 \pm 1^\circ\text{C}$), 4 mM sodium triphosphate, curve C; and 50 mM Tris (pH 7.4 at $20 \pm 1^\circ\text{C}$), 4 mM sodium ATP, curve D. Samples were brought to sedimentation equilibrium at 4°C . The data shown were obtained at 10,000 rpm; the smooth curves represent global fits of the monomer-dimer-n-mer model (Equation 1) to data obtained at 5,000, 7,000, 10,000, and 15,000 and for the samples in Tris alone, 20,000 rpm. The small amplitudes and uniform distributions of the residuals demonstrate the compatibility of this model with the data.

lated dynorphin B-9 and B-13 peptides were stimulated to a greater extent than the dynorphin A-related peptides.

The effect of ATP or triphosphate on the gross confirmation of IDE was examined by testing the effect of 4 mM ATP or 4 mM

PPP_i on the susceptibility of the enzyme to proteolysis by proteinase K. Preliminary experiments had shown that IDE was partially degraded by a 1-h incubation with proteinase K at a ratio of IDE/proteinase K of 1:200 (w/w). No change in the proteinase K cleavage pattern was seen in the presence of ATP or triphosphate, indicating the absence of a conformational change exposing proteinase K-sensitive sites. IDE is resistant to cleavage by trypsin and chymotrypsin at a 1:200 (w/w) ratio and remained resistant to these proteases in the presence of ATP and triphosphate. Thus there are no major structural changes that are produced by ATP or triphosphate that expose protease-sensitive sites.

ATP, Triphosphate, and Orthophosphate Affect the Oligomeric State of IDE—We found previously that IDE solutions in 100 mM sodium phosphate (pH 7.3 at 20 ± 1 °C) contain an equilibrium mixture of monomers, dimers, and tetramers, with monomers and tetramers the predominant species (9). To test the effects of phosphate compounds on oligomerization, we performed sedimentation analyses using solutions made in 50 mM Tris (pH 7.4 at 20 ± 1 °C). Samples equilibrated in Tris buffer alone had mass distributions consistent with mixtures of monomers and dimers, with no significant contribution from higher oligomers (corresponding to Equation 1 with $\alpha_{n,0} = 0$). The small, uniformly distributed residuals from this fit (Fig. 3, *curve A*) demonstrate the compatibility of the monomer-dimer model with the data. As found previously (9), samples equilibrated in 100 mM phosphate buffer gave mass distributions consistent with mixtures of monomers, dimers, and tetramers, (corresponding to Equation 1 with $n = 4$; Fig. 3, *curve B*). Most intriguingly, samples equilibrated in Tris containing 4 mM triphosphate had mass distributions consistent with mixtures of monomers (the dominant species) and high oligomers ($8 \leq n \leq 16$), but no detectable dimers (corresponding to Equation 1 with $\alpha_{d,0} = 0$; Fig. 3, *curve C*). Samples containing 4 mM ATP were more difficult to analyze because the absorbance of this compound interfered partially with detection of protein absorbance at 280 nm. However, a global analysis of data obtained at 5,000, 7,000, 10,000, and 15,000 rpm indicated that the monomer- n -mer model was appropriate, with n in the range $12 \leq n \leq 16$ (Fig. 3, *curve D*). Attempts to fit data obtained in the presence of ATP using the monomer-dimer- n -mer model (Equation 1) returned values for the absorbance contribution of the dimeric species ($\alpha_{d,0}$) that were within error equal to zero, consistent with the pattern obtained in the presence of 4 mM triphosphate.

DISCUSSION

Perhaps the first report of regulation of IDE activity by ATP was that of Hashimoto *et al.* (15) who found that ATP affected insulin degradation in rat hepatocytes. However, the study by Camberos *et al.* (10) was the first to report a direct effect of ATP on purified IDE. We have not been able to confirm that ATP has an inhibitory effect on insulin hydrolysis by IDE. However, we have found that ATP has a large stimulatory effect on the hydrolysis of relatively small peptide substrates for IDE. We further note that all nucleotide triphosphates and triphosphate itself produce similar effects. Our data suggest that the effect of ATP can be attributed to the triphosphate moiety and that even monophosphate itself can activate. These findings are most consistent with a model in which there is a cationic site on the enzyme that binds the phosphate moiety of a nucleotide or simply P_i , PP_i , or PPP_i . This cationic site is distinct from the substrate-binding site as shown by the ability of ATP and PPP_i to activate in the

presence of the substrate dynorphin B-9.

The sedimentation data shown here are consistent with a monomer-dimer- n -mer pattern of association with a continuum of n values ranging from 4 in phosphate buffer to ~16 in the presence of PPP_i and ATP. The observation of a common pattern with several different preparations of IDE and in both Tris and phosphate buffers strongly suggests that this association mechanism is a *bona fide* property of IDE and not an artifact of preparation or of our choice of reaction conditions. The significant differences in oligomerization found in the presence and absence of near-physiological concentrations of PPP_i and ATP raises the possibility that these, or related, compounds may regulate the oligomerization and activity states of IDE *in vivo*.

A likely mechanism for the polyanion effect derived from the sedimentation equilibrium experiments is that of shifting the monomer/dimer/tetramer equilibrium to the monomer. We have found that both ATP and PPP_i shift this equilibrium, as does phosphate to a lesser extent. The extent of monomer formation correlates with the increase in activity observed with small IDE substrates. The lack of a significant effect of a polyanion on larger peptide substrates, *i.e.* insulin and amyloid β peptide, could be interpreted to suggest that extended binding interactions that can occur with a larger substrate induce monomer formation in the absence of a polyanion. It is unlikely that the dimeric or tetrameric form of IDE favors hydrolysis of the larger substrates. If this were the case, polyanions would have acted as inhibitors of insulin and amyloid β peptide hydrolysis.

In summary, IDE appears to contain a cationic regulatory site that binds anions including nucleotide triphosphates primarily if not exclusively through their triphosphate moiety. The major effect of nucleotide triphosphates appears to be manifested through alteration of the oligomerization state of the enzyme toward a monomer, which increases activity toward small peptide substrates. Thus binding at the cationic site can alter the specificity of IDE by increasing its preference for small peptides.

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Enzyme Catalysis and Regulation: ATP Effects on Insulin-degrading Enzyme Are Mediated Primarily through Its Triphosphate Moiety

Eun Suk Song, Maria Aparecida Juliano, Luiz
Juliano, Michael G. Fried, Steven L. Wagner
and Louis B. Hersh

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