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Mutation of Active Site Residues of Insulin-degrading Enzyme Alters Allosteric Interactions*

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Eun Suk Song‡, Abigail Daily‡, Michael G. Fried‡, Maria Aparecida Juliano§, Luiz Juliano§, and Louis B. Hersh‡¶

From the ‡Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, Kentucky 40536 and the §Department of Biophysics, Escola Paulista de Medicina, Sao Paulo 04023-900, Brazil

The active site glutamate (Glu^{111}) and the active site histidine (His^{112}) of insulin-degrading enzyme (IDE) were mutated. These mutant enzymes exhibit, in addition to a large decrease in catalytic activity, a change in the substrate-velocity response from a sigmoidal one seen with the native enzyme (Hill coefficient > 2), to a hyperbolic response. With 2-aminobenzoyl-GGFLRKH-GQ-*N*-(2,4-dinitrophenyl)ethylenediamine as substrate, ATP and triphosphate increase the reaction rate of the wild type enzyme some 50–80-fold. This effect is dampened with glutamate mutants to no effect or less than a 3-fold increase in activity and changed to inhibition with the histidine mutants. Sedimentation equilibrium shows the IDE mutants exhibit a similar oligomeric distribution as the wild type enzyme, being predominantly monomeric, with triphosphate having little if any effect on the oligomeric state. Triphosphate did induce aggregation of many of the IDE mutants. Thus, the oligomeric state of IDE does not correlate with kinetic properties. The His^{112} mutants were shown to bind zinc, but with a lower affinity than the wild type enzyme. The glutamate mutants displayed an altered cleavage profile for the peptide β-endorphin. Wild type IDE cleaved β-endorphin at Leu^{17}-Phe^{18} and Phe^{18}-Lys^{19}, whereas the glutamate mutants cleaved at these sites, but in addition at Lys^{19}-Asn^{20} and at Met^{5}-Thr^{6}. Thus, active site mutations of IDE are suggested to not only reduce catalytic activity but also cause local conformational changes that affect the allosteric properties of the enzyme.

It is generally accepted that the accumulation of amyloid β-peptides (Aβ),[1] particularly amyloid β-peptide 1–42, plays a central role in the pathogenesis of Alzheimer disease (AD). The mechanisms leading to the accumulation of Aβ in late onset AD is currently unknown but could be a result of either overproduction of the peptide or decreased clearance. In the case of autosomal dominant AD, it is clear that the early onset of the disease is linked to increased Aβ production and results from mutations in one of several genes: *APP*, *PS1* (presenilin 1), or *PS2* (presenilin 2) (1–4). However, there is only a scattering of evidence that late onset AD is attributable to an overproduction of Aβ (5). There are recent reports (6–8) that the β-secretase involved in amyloid β-peptide synthesis is increased in sporadic AD patients. Whether this increase is a cause of or a consequence of AD and whether this increase in activity will be seen in larger patient populations remains to be established.

Clearance of Aβ is currently receiving considerable attention as a key regulatory mechanism of brain Aβ levels. There appear to be two paths for Aβ clearance in the brain. One pathway involves the equilibration of Aβ between the central nervous system and plasma mediated through cerebral spinal fluid (9–11). The other pathway involves clearance of Aβ through proteolysis (12–15). There are a number of peptidases that have been implicated in Aβ clearance. Those that have received the most attention and are supported by *in vivo* studies in peptidase-deficient mice include neprilysin (16, 17), insulysin (IDE) (18, 19), and endothelin-converting enzyme (20, 21). Genetic studies have further suggested a linkage between late onset AD and IDE. A linkage of a region of chromosome 10q to late onset Alzheimer disease has been reported by several groups (22–25). This region of the chromosome is within 195 kilobases of the IDE gene. However, to date this analysis has not been refined to the point where the linkage to IDE can be unequivocally established.

IDE (EC 3.4.24.56) is an ~110-kDa zinc metallopeptidase containing an inverted form of the signature active site sequence (H*XX*EH) and is thus known as an inverzincin. The enzyme is primarily cytosolic, although a fraction of the enzyme is peroxisomal due to the presence of a C-terminal peroxisomal targeting sequence (26). IDE has also been reported as being located on the cell surface and secreted (27–29). Farris *et al.* (30) have shown that naturally occurring IDE missense mutations in the rat result in decreased insulin and Aβ degradation. Primary neuronal cultures derived from these rats secrete increased levels of Aβ peptides; however, the steady-state level of brain Aβ in these animals is unaffected, suggesting compensatory mechanisms.

The specificity of IDE is complex. The enzyme cleaves a variety of peptides primarily, but not exclusively, at basic or bulky hydrophobic residues (31, 32). Kurochkin (33) has suggested that IDE cleaves peptides that have a propensity to form β-pleated sheet structures. Cleavage of Aβ by IDE results in the loss of the neurotoxic effects of the peptide and prevents the deposition of Aβ onto a synthetic amyloid deposit (34). We have recently shown that IDE exhibits allosteric properties being

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¶ To whom correspondence should be sent: Dept. of Molecular and Cellular Biochemistry, College of Medicine, University of Kentucky, 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: Aβ, amyloid β-peptide(s); PPP_i, tripolyphosphate; AD, Alzheimer disease; IDE, insulin-degrading enzyme; HPLC, high pressure liquid chromatography; Abz, 2-aminobenzoyl; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine.

TABLE I
Oligonucleotides used

Oligonucleotide	Sequence
H112Q-forward	5′-AGCCATTTTTGTGAGCAGATGCTGTTTTTGGGAAC-3′
H112Q-reverse	5′-GTTCCCAAAAACAGCATCTGCTCACAAAAATGGCT-3′
H112D-forward	5′-AGCCATTTTTGTGAGGATATGCTGTTTTTGGGAAC-3′
H112D-reverse	5′-GTTCCCAAAAACAGCATATCCTCACAAAAATGGCT-3′
E111A-forward	5′-CTTAAGCCATTTTTGTGCGCATATGCTGTTTTTGG-3′
E111A-reverse	5′-CCAAAAACAGCATATGCGCACAAAAATGGCTTAAG-3′
E111V-forward	5′-CTTAAGCCATTTTTGTGTGCATATGCTGTTTTTGG-3′
E111V-reverse	5′-CCAAAAACAGCATATGCACACAAAAATGGCTTAAG-3′
E111L-forward	5′-CTTAAGCCATTTTTGTTTGCATATGCTGTTTTTGG-3′
E111L-reverse	5′-CAAAAACAGCATATGCAAACAAAAATGGCTTAAG-3′
E111F-forward	5′-CTTAAGCCATTTTTGTTTCCATATGCTGTTTTTGG-3′
E111F-reverse	5′-CCAAAAACAGCATATGGAAACAAAAATGGCTTAAG-3′

activated by peptide substrates. Interestingly, peptides activated IDE toward Aβ peptide cleavage but not toward insulin cleavage (35). We further established that IDE exists as a mixture of monomers, dimers, and tetramers. During the course of our studies on the allosteric properties of IDE, we generated a number of active site mutants as control enzymes. We noted that although the activity of these mutants was significantly reduced, the residual catalytic properties were altered. We report here the results of these studies.

MATERIALS AND METHODS

Synthesis of the fluorogenic peptide substrate Abz-GGFLRKHGQ-EDDnp was as previously described (36). Dynorphin A-9, dynorphin A-10, dynorphin A-17, dynorphin B-9, and insulin were obtained from Bachem. Amyloid β peptide 1–40 was a product of California Research Peptide Inc. β-endorphin was obtained from Multiple Peptide Systems through the National Institute on Drug Abuse Research Tools program. All other reagents were obtained as the best grade available commercially.

Activity Assays—IDE activity was routinely measured with the fluorogenic peptide Abz-GGFLRKHGQ-EDDnp. The increase in fluorescence that occurred upon cleavage of the peptide between residues R and K (36) was followed on a SpectraMax Gemini XS fluorescence plate reader using an excitation wavelength of 318 nm and an emission wavelength of 419 nm.

The IDE-dependent hydrolysis of insulin, β-endorphin, amyloid β-peptide 1–40, and dynorphin peptides was followed by reverse phase HPLC using a C4 column and a linear gradient from 0.1% trifluoroacetic acid in 95% water, 5% acetonitrile to 0.1% trifluoroacetic acid in 50% water, 50% acetonitrile. Peptides were followed by their absorbance at 214 nm and quantified from their peak area.

Preparation and Purification of IDE Mutants—The various His^{112} and Glu^{111} rat IDE mutants were prepared in pFast Bac HTb using the site-directed mutagenesis kit from Stratagene. (It should be noted that a methionine located 42 residues downstream of methionine 1 of the IDE cDNA appears to represent the major translational start site (37). Thus, His^{112} would actually be His^{70}, and Glu^{111} would be Glu^{69}. We have however retained the original numbering system to avoid confusion with earlier publications). The oligonucleotides used for mutagenesis, with the base changes from the wild type sequence underlined, are listed in Table I.

Based on the secondary structure predicting programs PHD (38) and PHDsec (39), IDE is predicted to have a helical region from residues 107 to 116, which encompasses the mutated residues. None of the mutations were predicted to affect the ability of this region to form a helix.

Recombinant IDE and the various mutants were expressed in Sf-9 cells as a fusion protein containing an N-terminal hexahistidine affinity tag followed by a linker containing a tobacco etch virus protease cleavage site (35). Wild type and IDE mutants were purified on a His-Select HC nickel affinity gel column (Sigma). In most experiments, the enzyme was used as the His_6-IDE; however, to verify the lack of an effect of the N-terminal hexahistidine and linker, tobacco etch virus protease was used to remove this region as previously described (35).

Sedimentation Equilibrium Analysis—Analytical ultracentrifugation was performed at 4.0 ± 0.1 °C in a Beckman XL-A centrifuge using an AN 60 Ti rotor. Scans were obtained at 280 nm with a step size of 0.001 cm. The approach to equilibrium was considered to be complete when scans made 6 h apart were indistinguishable. Typically, equilibration times equal to or greater than 20 h met this criterion. Five scans were averaged for each sample at each rotor speed. Previous results (3) indicated that IDE self-associates in a monomer-dimer-tetramer pattern. Sedimentation equilibrium data were therefore analyzed according to Equation 1,

$$A(r) = \alpha_{m,0}\exp(\sigma_m(r^2 - r_o^2)) + \alpha_{d,0}\exp(2\sigma_m(r^2 - r_o^2)) + \alpha_{t,0}\exp(4\sigma_m(r^2 - r_o^2)) + \varepsilon \quad \text{(Eq. 1)}$$

where $A(r)$ is the absorbance at radial position r, and $\alpha_{m,0}$, $\alpha_{d,0}$, and $\alpha_{t,0}$ are the absorbances of monomer, dimer, and tetramer, respectively, at the reference radius (r_o). 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 partial specific volume; ρ is the solvent density, ω is the rotor angular velocity, R is the gas constant, T is the absolute temperature, and ϵ is the base-line offset. 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 (40). Data sets obtained for samples run at three different rotor speeds were fit simultaneously to Equation 1 using the global fitting method first described by Johnson and co-workers (41).

RESULTS

IDE is a zinc metallopeptidase of the inverzincin family containing the signature active site sequence $H^{108}XXEH$. The two histidines, in conjunction with a downstream glutamate (Glu^{189}) are believed to act as ligands for the active site zinc (37, 42). As with other zincins, glutamate 111 is believed to be directly involved in catalysis, most probably acting as a general base to facilitate removal of a proton from a water molecule as it attacks the scissile bond. The effect of mutating the active site glutamate and histidine has been reported to produce an inactive enzyme (37, 42). Since we noted varying extents of inactivation when mutating similar residues in the related zinc metallopeptidase puromycin-sensitive aminopeptidase (43), we quantitated the effect of mutating His^{112} and Glu^{111} of IDE.

Two mutants of His^{112}, H112Q and H112D, were generated as well as four mutants of Glu^{111}: E111V, E111L, E111A, and E111F. We initially compared the kinetics of the various His^{112} and Glu^{111} mutants using the fluorogenic substrate Abz-GGFLRKHGQ-EDDnp. Although native IDE clearly displayed a sigmoidal substrate binding curve (Fig. 1), both of the His^{112} mutants and all four of the Glu^{111} mutants displayed classical hyperbolic substrate binding curves as illustrated for the E111F mutant in Fig. 1. Table II summarizes the kinetic parameters for each of the mutants. Both of the His^{112} mutants exhibited a 3–5-fold increase in K_m, with k_{cat} decreased close to 7,000-fold for the H112D mutant but less than 100-fold for the H112Q mutant. Mutation of the active site glutamate to valine, leucine, alanine, or phenylalanine produced enzyme forms in which the effects on K_m were variable from essentially no change for the E111L and E111F mutants to a greater than 7-fold increase for the E111A mutant. The range of k_{cat} values for the Glu^{111} mutants varied from a decrease of ~9,000-fold (E111A) to a decrease of ~68,000-fold (E111L).

We also examined the effect of the Glu^{111} and His^{112} mutations on the hydrolysis of three physiological peptide substrates for IDE, insulin, β-endorphin, and amyloid β peptide 1–40. As shown in Table III, under saturating substrate conditions, β-endorphin and amyloid β peptide 1–40 hydrolysis was decreased ~10^7-fold for the Glu^{111} mutants and ~10^6-fold for the His^{112} mutants. With insulin as substrate, no hydrolysis could be detected with the Glu^{111} mutants under conditions in which a decrease of ~10^7-fold could have been detected, whereas with the His^{112} mutants, insulin hydrolysis was decreased 3×10^6-fold (H112Q) to 10^7-fold (H112D).

IDE has been found to contain a cationic regulatory site distinct from the active site, which binds nucleotide triphosphates primarily through the triphosphate moiety (44). The

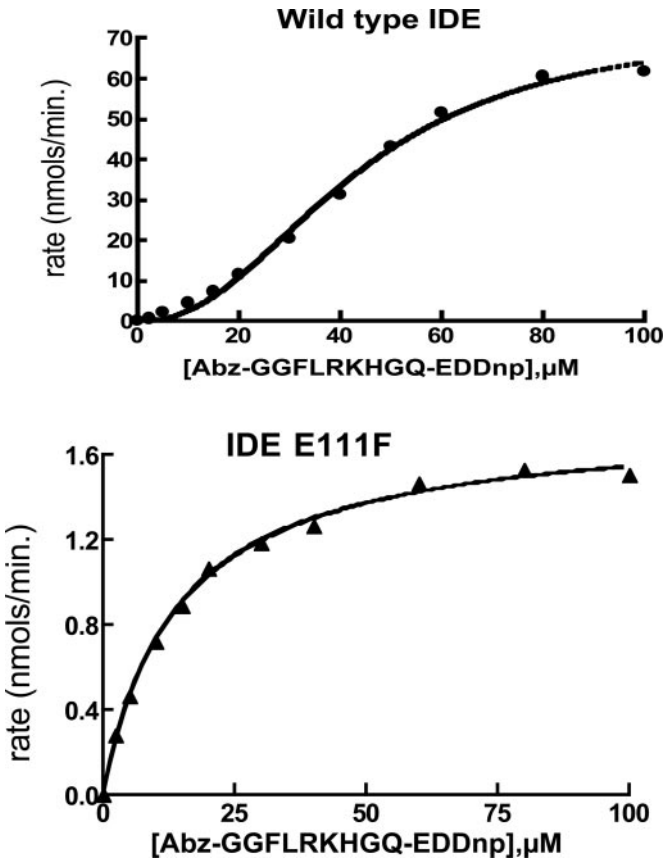

FIG. 1. **Comparison of the kinetics of wild type IDE with the kinetics of the E111F mutant.** Reactions were conducted in 50 mM Tris-HCl, pH 7.4, with wild type IDE (50 ng of enzyme) (*top*) or the E111F mutant (30 μg of enzyme) (*bottom*) using Abz-GGFLRKHGQ-EDDnp as the variable substrate.

TABLE II
Kinetic properties of IDE mutants with Abz-GGFLRKHGQ-EDDnp as substrate

Activity was determined in 20 mM potassium phosphate buffer, pH 7.3.

Mutant IDE	K_m or $K_{0.5}$	k_{cat}	Hill coefficient
	μM	s^{-1}	
Wild type	28.7 ± 3.0	162,580 ± 9036	2.26 ± 0.2
H112Q	158.2 ± 37.8	1,953 ± 330.0	1.10 ± 0.17
H112D	83.2 ± 2.9	23.7 ± 0.5	1.00 ± 0.03
E111V	15.6 ± 1. 9	2.4 ± 0.1	0.72 ± 0.11
E111L	14.0 ± 0.8	6.5 ± 0.1	0.97 ± 0.07
E111A	204.0 ± 30.8	17.5 ± 2.0	0.89 ± 0.08
E111F	27.4 ± 2.9	11.4 ± 4.8	0.75 ± 0.07

binding of a polyanion at this site increases the rate with small peptides as substrates, but not with the larger physiological substrates insulin or amyloid β peptide. Since the active site histidine and glutamate mutations caused a loss of allosteric interactions, we tested whether the mutant enzymes could still be activated by ATP and triphosphate. As shown in Table IV, using Abz-GGFLRKHGQ-EDDnp or dynorphin B-9 as substrate, the Glu^{111} mutants were activated by ATP to a much lesser extent compared with the wild type enzyme. A similar effect was observed with triphosphate as the activator.

The His^{112} mutants were affected differently by ATP and triphosphate, which acted as inhibitors rather than activators. With the H112Q mutant, triphosphate was an effective inhibitor of Abz-GGFLRKHGQ-EDDnp hydrolysis at micromolar concentrations (K_i^{PPPi} = 2.3 μM; K_i^{ATP} = 2.2 μM), whereas activation of the wild type enzyme required millimolar concentrations (K_A^{PPPi} = 1.7 mM, K_A^{ATP} = 1.0 mM); this is illustrated in Fig. 2.

TABLE III
Rates of hydrolysis of physiological peptides by IDE mutants

Activity was determined in 50 mM Tris-HCl buffer, pH 7.4, with 10 μM peptide. Rates of hydrolysis were determined by following substrate disappearance by HPLC.

Mutant IDE	β-Endorphin	Aβ 1–40	Insulin
		pmol/min/ng	
Wild type	315,000	420,000	594,000
H112Q	0.335	0.292	0.183
H112D	0.263	0.191	0.052
E111V	0.049	0.074	ND[a]
E111L	0.060	0.022	ND
E111A	0.025	0.105	ND
E111F	0.027	0.032	ND

[a] ND, not detectable.

We used sedimentation equilibrium to determine what if any effect the active site mutations had on IDE oligomerization (Fig. 3). We previously reported that in Tris buffer, the wild type enzyme appeared predominantly as a mixture of monomers and dimers (44). The monomer was the predominant species. The addition of 4 mM triphosphate maintained the monomer as the dominant species but eliminated the small amount of dimers (44). In this study, we found that the H112Q mutant appeared monomeric with a trace of a higher aggregate, possibly the tetramer, present. The addition of PPP_i decreased solubility but did not significantly change the distribution of oligomeric species. In contrast, the H112D mutant appeared less soluble but showed predominantly tetramers. The addition of PPP_i decreased solubility but clearly showed the presence of primarily monomers with higher aggregates present. E111A, E111F, and E111L all appeared as monomers with a trace of tetramer present. E111V exhibited poor solubility and could not be analyzed. The addition of PPP_i to E111L and E111A had no effect on the distribution of species but induced some aggregation with E111A and complete aggregation of E111F. Thus, there is no clear correlation between oligomeric state and enzyme activity.

Perlman *et al.* (42) showed that His^{112} is a zinc-coordinating residue and provided evidence that the active site zinc was lost in the H112Q mutant. We reexamined this issue with both the H112Q and H112D mutants. To accomplish this we looked at the ability of EDTA and *o*-phenanthroline to inhibit the residual activity of these mutants. As shown in Table V, inclusion of 0.1 mM EDTA or 0.1 mM *o*-phenanthroline in the assay had little effect on the activity of wild type IDE. Similarly, 0.1 mM EDTA or 0.1 mM *o*-phenanthroline had no effect on the rate of insulin hydrolysis by the glutamate mutants (data not shown). In contrast, both metal-chelating agents completely inhibited the activity of the histidine mutants as shown in Table V. Although not shown, significant inhibition of the wild type enzyme and glutamate mutants was observed with higher concentrations of EDTA (1 mM) and *o*-phenanthroline (1 mM). Thus, the His^{112} mutants appear to retain active site zinc, but this zinc is bound more weakly. We thus looked at the ability of zinc to increase the reaction rate of the His mutants. As shown in Fig. 4, the addition of zinc over the concentration range of 0.1–10 μM caused a maximal ~1.8-fold increase in the activity of the H112Q mutant and a ~1.2-fold increase in the activity of the H112D mutant. This was followed by inhibition at higher zinc concentrations. In contrast, zinc, at all concentrations tested, was slightly inhibitory of the wild type activity.

In order to determine whether the mutations introduced into IDE affected enzyme specificity, we looked at the cleavage pattern of three physiological peptides, β-endorphin, insulin, and amyloid β peptide 1–40 using each of the mutants listed in

TABLE IV
Effect of ATP and PPP_i on mutant IDE activity

Activity was determined with 10 μM Abz-GGFLRKHGQ-EDDnp or 10 μM dynorphin B-9 in 50 mM Tris-HCl buffer, pH 7.4, in the presence of 5 mM ATP or 5 mM PPP_i and compared with that in the absence of any addition. Specific activities in the absence of added ATP or PPP_i with Abz-GGFLRKHGQ-EDDnp as substrate were as follows: wild type IDE = 8,131 nmol/min/mg; E111A, 7.5 nmol/min/mg; E111V, 2.7 nmol/min/mg; E111F, 5.2 nmol/min/mg; E111L, 0.092 nmol/min/mg; H112D, 28.1 nmol/min/mg; H112Q, 847 nmol/min/mg.

IDE form	Relative activity			
	Abz-GGFLRKHGQ-EDDnp		Dynorphin B-9	
	ATP	PPP_i	ATP	PPP_i
Wild type	79.6	54.0[a]	29	29
H112Q	0.20[b]	0.007[b]	0.42	0.41
H112D	0.89	0.21	0.84	0.27
E111A	2.53	1.45	2.3	6.2
E111L	1.07	1.15	2.8	2.1
E111F	2.81	1.75[a]	3.0	1.9
E111V	0.94	0.67	1.7	3.5

[a] Measured at 1 and 2.5 mM PPP_i as inhibition occurred at 5 and 10 mM.
[b] Measured at 10 mM ATP and PPP_i as inhibition was observed at all concentrations.

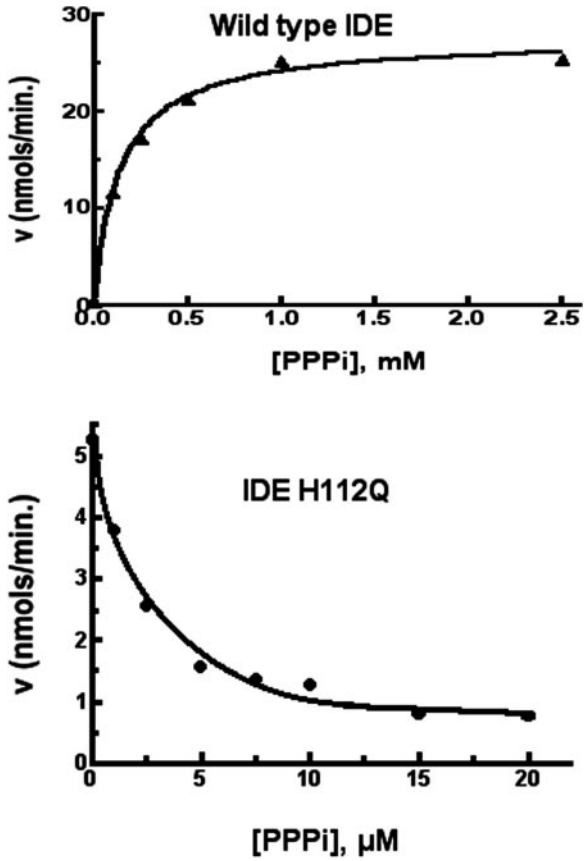

FIG. 2. **Comparison of the effect of triphosphate on the hydrolysis of Abz-GGFLRKHGQ-EDDnp by wild type IDE (*top*) with the IDE H112Q mutant (*bottom*).** Activity was determined in 50 mM Tris-HCl buffer, pH 7.4, with 10 μM Abz-GGFLRKHGQ-EDDnp as substrate and the indicated concentration of PPP_i. The reactions with wild type enzyme contained 50 ng of protein, whereas those with the H112Q mutant contained 10 μg of protein. For the wild type enzyme, the activity in the absence of added PPP_i was 0.35 nmol/min. Although not shown, there was no effect of PPP_i on the activity of the wild type enzyme over the same concentration range used for the H112Q mutant.

Table II. With insulin and amyloid β peptide 1–40 as substrate, we observed the same initial cleavage products with the various mutants and wild type enzyme. This is illustrated for insulin cleavage by the E111F mutant in Fig. 5**.** As previously

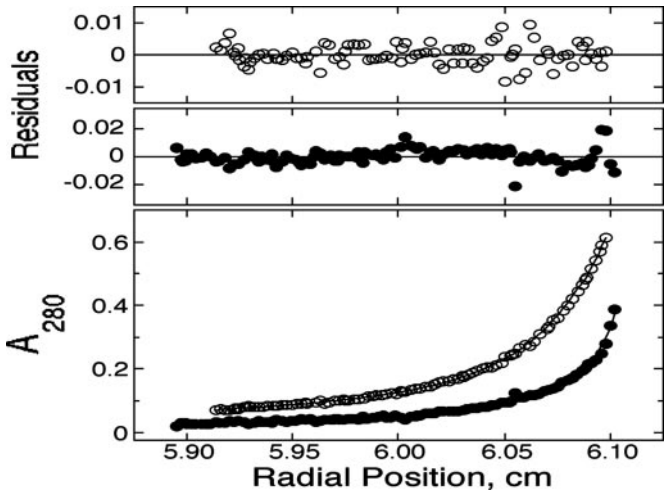

FIG. 3. **Sedimentation equilibrium analysis of wild type and E111A mutant IDE proteins.** Samples were dissolved in 50 mM Tris-HCl buffer, pH 7.4, and brought to sedimentation equilibrium at 10,000 rpm and 4 °C. *Open circles*, wild type IDE; *filled circles*, E111A mutant IDE. The *smooth curves* represent global fits of Equation 1 to data sets obtained at 10,000, 15,000, and 20,000 rpm. The small, symmetric residuals demonstrate the compatibility of this equation with the data. The data for both the wild type and mutant IDE are consistent with mixtures in which monomeric enzyme predominates over relatively small mol fractions of higher oligomers.

TABLE V
Effect of metal chelators on mutant IDE activity

Activity was determined in 50 mM Tris-HCl, pH 7.4, with either 10 μM Abz-GGFLRKHGQ-EDDnp as substrate or 10 μM insulin (values in parenthesis) as substrate and 0.1 mM EDTA or 0.1 mM *o*-phenanthroline added as indicated.

IDE form	Percentage of activity remaining	
	EDTA	*o*-phenanthroline
	%	%
Wild type	90.8 (123)	73.2 (142)
H112Q	<0.2 (<1)	<0.1 (16)
H112D	<2.5 (50)	<2.5 (60)

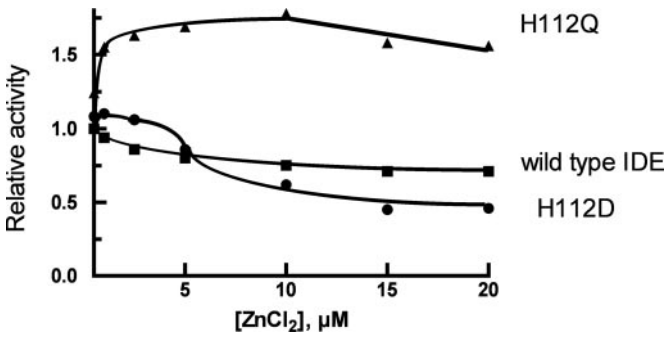

FIG. 4. **The effect of zinc on the activity of wild type IDE and its His^{112} mutants.** Activity was determined in 50 mM Tris-HCl buffer, pH 7.4, with 10 μM Abz-GGFLRKHGQ-EDDnp as substrate and the indicated concentration of $ZnCl_2$.

noted (32), native IDE cleaved β-endorphin at the Thr^{17}-Leu^{18} and Leu^{18}-Phe^{19} bonds, and the two histidine mutants produced the same cleavage pattern. However, each of the glutamate 111 mutants produced, in addition to cleavage at Leu^{17}-Phe^{18} and Phe^{18}-Lys^{19}, cleavage at Lys^{19}-Asn^{20} and at Met^{5}-Thr^{6}. This is illustrated in Fig. 5 for the E111F mutant. Based on this result, we also compared the cleavage of small dynorphin-related peptides by native IDE and by the E111V mutant. We found that with dynorphin A-9, dynorphin A-10, dynorphin A-17, and dynorphin B-9, there was no change in cleavage site.

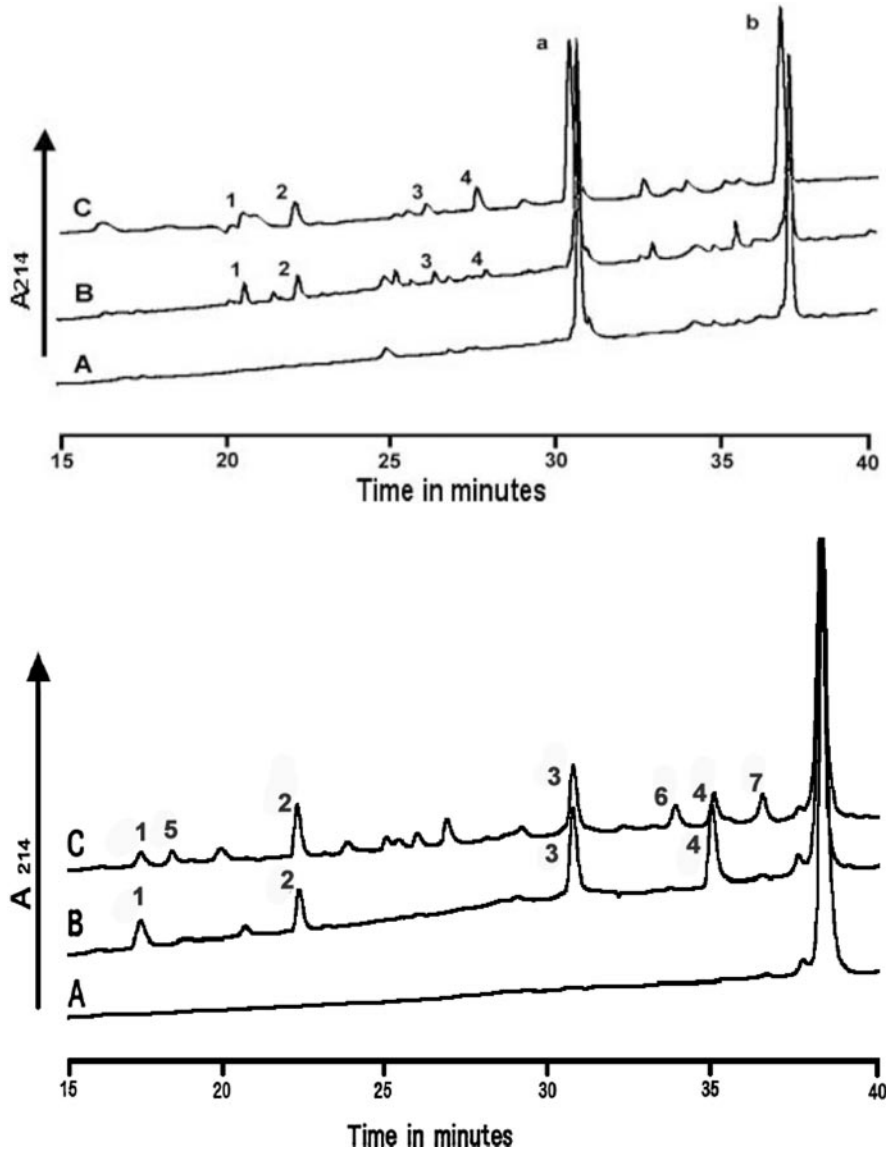

FIG. 5. **HPLC chromatograms showing the cleavage of β-endorphin and insulin by IDE and its E111F mutant.** *Top*, reaction mixtures containing 10 μM insulin in 50 mM Tris-HCl, pH 7.4 (*A*), were reacted with wild type IDE (0.1 μg for 30 min) (*B*) or E112Q (50 μg for 1 h) (*C*). Reaction products were separated by gradient HPLC on a Vydac C4 reverse phase column as described under "Materials and Methods." Product peaks were collected and identified by mass spectrometry. *Peak 1*, insulin B1–9; *peak 2*, insulin B1–10; *peak 3*, insulin A1–13; *peak 4*, insulin A1–14; *peak a*, insulin A chain; *peak b*, insulin B chain. *Bottom*, reaction mixtures as above containing 10 μM β-endorphin (*A*) reacted with wild type IDE (0.1 μg for 15 min) (*B*) or E111F (50 μg for 1 h) (*C*). *Peak 1*, β-endorphin 19–31; *peak 2*, β-endorphin 18–31; *peak 3*, β-endorphin 1–17; *peak 4*, β-endorphin 1–18; *peak 5*, β-endorphin 20–31; *peak 6*, β-endorphin 1–19; *peak 7*, β-endorphin 6–31.

DISCUSSION

Mutation of residues within the active site motif (HE*XX*H or H*XX*EH) of zinc metallopeptidases has been reported to lead to a loss of catalytic activity. However, in most cases, the presence of residual activity has not been quantified, generally because of the lack of availability of large amounts of mutant protein. In most cases, the active site glutamate has been mutated (45–49), and although the mutant enzyme is reported to be catalytically inactive, when determined, the inactive enzyme retains the ability to bind substrate (37, 42, 43, 45).

The active site glutamate (Glu^{111}) and the active site histidine (His^{112}) of IDE have previously been mutated (37, 42). The His^{112} mutant transiently expressed in COS cells appeared to be inactive as measured in crude extracts with insulin as substrate. However, this mutant retained the ability to bind insulin as determined by cross-linking studies (37). It should be noted that insulin cross-linking was actually increased with the mutant relative to the wild type enzyme and was attributed to a "relaxation of a rigid conformation stabilized by zinc." Evidence for this increased flexibility at the active site came from the demonstration that treatment of the native enzyme with *o*-phenanthroline increased the extent of cross-linking; however, *o*-phenanthroline did not increase cross-linking with the H112Q mutant.

Using radioactive zinc, it was concluded that the H112Q mutant did not bind zinc. Our finding that the residual activity of the two His^{112} mutants, but not the Glu^{111} mutants, can be further reduced by treatment with metal chelators indicates that these mutants do contain zinc but that the zinc is bound considerably more weakly than with the native protein. Thus, our results with both the His^{112} and Glu^{111} mutants support and extend the general conclusions of Perlman and Rosner (37) that mutation of the active site histidine causes a change in the conformation about the active site of IDE. The results presented here suggest that this is not due to the loss of zinc binding but probably due to local conformational changes that occur within the active site.

A rather surprising finding is that mutation of either the catalytic glutamate or the zinc coordinating histidine converts the substrate-velocity curves from sigmoidal to hyperbolic. Thus, the homotropic allosteric activation previously reported by us (35) is absent in the mutants. Since it has been established that IDE can exist in an oligomeric state (32, 35, 50, 51), one possible explanation is that the active site of IDE is at the interface of two subunits and that mutation of the active site residues produces a local conformational change that affects subunit interaction. This does not appear to be the case, since both we (35) and Perlman *et al.* (42) have shown that formation

of oligomers containing active and inactive subunits does not abrogate activity. However, such a mixed oligomer does eliminate allosteric activation (35). Thus, the data is probably explained by the proposal that the active site mutations produce a local conformational change that is transmitted to the adjacent subunit. Although the end result is the same, it appears that there are differences in the local conformational changes produced by mutating the catalytic glutamate and mutating the zinc-coordinating histidine. This is most evident from the observation that the cleavage profile for β-endorphin is altered in the glutamate mutants but not the histidine mutants.

In addition to the active site mutations affecting the kinetics of substrate binding, these mutations also desensitized the enzyme to activation by triphosphates in the form of ATP or triphosphate itself. The large increase in activity seen with the native enzyme was significantly reduced to either no effect or less than a 3-fold increase in activity with the glutamate mutants. Even more amazing was the effect seen with the active site histidine mutants, particularly H112Q. In this case, triphosphate changed from an activator to a rather potent inhibitor. This clearly indicates that the active site of these mutants is conformationally different from the wild type enzyme and is further modulated by additional conformational changes produced by the binding of the triphosphate moiety.

The sedimentation equilibrium data show that, with the exception of the H112D mutant, both the native enzyme and the mutants are predominantly monomeric. Triphosphate does not affect the qualitative oligomerization mechanism of the soluble enzyme, but it increased the tendency to form insoluble aggregates in the case of the E111A and E111F mutants. Such aggregation may reflect a conformational change due to triphosphate binding. Thus, there is no obvious correlation between oligomeric state and activity, and the finding of a monomeric native enzyme is not consistent with its allosteric properties. On the other hand, gel filtration shows the native enzyme existing as dimers and tetramers, and at higher protein concentrations dynamic light scattering shows exclusively tetramers. Thus, it is clear that IDE can exist in a number of different oligomerization states. We would suggest that the allosteric kinetics observed can result from substrate induced oligomerization. In the case of mutant IDE, perhaps an altered local conformational change at the active site is transmitted through the protein such that it prevents the formation of oligomers.

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Enzyme Catalysis and Regulation:

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Eun Suk Song, Abigail Daily, Michael G. Fried, Maria Aparecida Juliano, Luiz Juliano and Louis B. Hersh

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