Specific Inhibition and Stabilization of Aspergilloglutamic Peptidase by the Propeptide

IDENTIFICATION OF CRITICAL SEQUENCES AND RESIDUES IN THE PROPEPTIDE*

Received for publication, September 21, 2004, and in revised form, October 28, 2004
Published, JBC Papers in Press, October 30, 2004, DOI 10.1074/jbc.M410852200

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Aspergilloglutamic peptidase (formerly called aspergillopepsin II) is an acid endopeptidase produced by Aspergillus niger var. macrosporus, with a novel catalytic dyad of a glutamic acid and a glutamine residue, thus belonging to a novel peptidase family G1. The mature enzyme is generated from its precursor by removal of the putative 41-residue propeptide and an 11-residue intervening peptide through autocatalytic activation. In the present study, the propeptide (Ala1–Asn41) and a series of its truncated peptides were chemically synthesized, and their effects on the enzyme activity and thermal stability were examined to identify the sequences and residues in the propeptide most critical to the inhibition and thermal stabilization. The synthetic propeptide was shown to be a potent competitive inhibitor of the enzyme ($K_i = 27 \text{ nM}$ at pH 4.0). Various shorter propeptide fragments derived from the central region of the propeptide had significant inhibitory effect, whereas their Ala scan-substituted peptides, especially R19A and H20A, showed only weak inhibition. Substitution of the Pro28–Pro34 sequence near His30 with an Ala–Ala sequence changed the peptide Lys18–Tyr25 to a substrate with His20 as the P1 residue. Furthermore, the propeptide was shown to be able to significantly protect the enzyme from thermal denaturation ($\Delta T_m = -19 \degree C$ at pH 5.6). The protective potencies of the propeptide as well as truncated propeptides and their Ala scan-substituted peptides are parallel with their inhibitory potencies. These results indicate that the central part, and especially Arg19 and His20 therein, of the propeptide is most critical to the inhibition and thermal stabilization and that His20 interacts with the enzyme at or near the S1 site in a nonproductive fashion.

Propeptides are known to be present in many protein precursors mostly at their N termini and are assumed to play various roles in the proteins such as inhibition of protein functions, stabilization of precursor structures, promotion of polypeptide folding, and/or intercellular protein sorting and targeting (1–6). These roles, however, have not yet been fully understood, and further studies on various proteins are necessary. As for the inhibitory properties of the propeptides of peptidases, there are numbers of reports describing their inhibition profiles and type of inhibition (7–25). However, only a few attempts have been made so far to identify critical sequences and/or residues in the propeptide important for inhibition of the corresponding mature peptidase, and much of the inhibition mechanisms, the structural determinants in the propeptide, and the sites of binding involved in the inhibition remains to be established. On the other hand, there are few studies that analyzed the effects of propeptide and its truncated fragments on the stability of the mature enzyme to identify critical sequences and/or residues contributing to the enzyme stability.

Aspergilloglutamic peptidase (AGP1; MEROPS ID: G01.002; formerly called aspergillopepsin II) is a pepstatin-insensitive acid endopeptidase produced by the fungus Aspergillus niger var. macrosporus (26). Like scytalidoglutamic peptidase (or eqolisin; MEROPS ID: G01.001; formerly called scytalidopepsin B) (27, 28), AGP also belongs to the newly established family of glutamic peptidases (i.e. peptidase family G1). The enzyme has two essential residues, a glutamic acid and a glutamine residue, at the active site (29–32), which is thought to form a catalytic dyad, and is not inhibited by any of the known inhibitors for aspartic, cysteine, metallo-, and serine peptidases. The enzyme is synthesized as a 282-residue proenzyme, and after removal of the 18-residue putative signal peptide, the proform is converted autocatalytically to the two-chain mature enzyme (composed of a 39-residue light chain and a 173-residue heavy chain) by liberation of the putative 41-residue propeptide (Ala1–Asn41) and the 11-residue intervening peptide (33, 34). So far, no study has been carried out on the roles of the propeptide in glutamic peptidases, including their inhibitory and stabilizing potencies. Therefore, it is important to clarify their roles, which should also help to develop specific inhibitors for the enzyme.

As the first step in this direction, we chemically synthesized, in the present study, the propeptide and its various truncated fragments and investigated their effects on the activity and thermal stability of the mature enzyme. As a result, the propeptide was shown not only to competitively inhibit the enzyme activity but also to significantly stabilize the enzyme against thermal denaturation. Comparison of the effect of inhibition with that of thermal stabilization by a series of the truncated propeptides revealed that the central part (Lys18–Tyr25), especially Arg19 and His20 therein, is most important for both these effects. This is the first time that a short peptide segment and some residues therein of the propeptide of a

* This study was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, Culture, and Sports of Japan and the Japan Society for Promotion of Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: AGP, aspergilloglutamic peptidase; DSC, differential scanning calorimetry; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HPLC, high performance liquid chromatography; MES, 4-morpholineethanesulfonic acid.

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peptidase were shown to be critical to both enzyme inhibition and stability.

**EXPERIMENTAL PROCEDURES**

**Materials**—The culture filtrate powder (Proctase) of *A. niger var. macrosporus* was kindly supplied by Meiji Seika Co., (Tokyo). AGP was purified from Proctase by column chromatography on DEAE-Toyopearl 6505 (Tosoh) and Sephacryl S-100 (Amersham Biosciences) essentially as described previously (35), except that the enzyme was eluted from the DEAE-Toyopearl column with the pH 2.5 buffer after washing with the pH 3.5 buffer. Substance P was purchased from Sigma, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was from Pierce. All other chemicals used were of analytical grade and were obtained from Wako Pure Chemicals Ind.

**Synthesis of the Propeptide and Its Fragments**—Peptides were synthesized manually by the conventional Fmoc solid phase peptide synthesis method (36). The structure of each peptide was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis (ToFSpecE, Micromass, Ltd.), and its amount was determined by amino acid analysis after acid hydrolysis (6 N HCl, 150 °C, 2 h) using a PerkinElmer Life Sciences model 421 automatic amino acid analyzer.

**Enzyme Assay and Determination of Kinetic Constants**—The activity of the enzyme was measured with substance P as substrate. Under the standard conditions, the enzyme (40 nm) and substance P (200 μM) were incubated at 30 °C in 30 mM sodium acetate buffer, pH 4.0. The reaction was stopped by the addition of 100 μl of 200 mM sodium phosphate buffer, pH 8.0. A 100-μl aliquot from the reaction mixture was analyzed by reverse-phase HPLC using a Shimadzu LC10A system on a Tosoh ODS-120T column (4.6 × 250 mm) eluted with an acetonitrile gradient (0–60%) in 0.1% trifluoroacetic acid and monitored at 215 nm. The initial velocity was estimated from the increase in the amount of the hydrolysis products at 17 time points (0–480 s). The reaction was stopped by the addition of 100 μl of the hydrolysis products at 17 time points (0–480 s). The Km and Vmax values were estimated from Lineweaver-Burk plots at 12 different substrate concentrations (1–1000 μM). The IC50 values were measured in the presence of 200 μM substance P and 0–200 nM peptides (6–24 different concentrations). The Km values were estimated from Dixon plots or from the IC50 values with the Cheng and Prusoff equation: Km = IC50/[1 + (S/Km)] (37).

**Measurement of the Effect of pH on the Propeptide Inhibition**—The enzyme (40 nm) and substance P (200 μM) were incubated at 30 °C for 2 min in 30 μl of buffers, pH 0.4–8.0, in the presence or absence of 150 mM propeptide Ala1–Asn41. The buffers used were: 100 mM KCl/HCl buffers, pH 0.4–2.3; 100 mM glycine/HCl buffers, pH 2.6–3.5; 100 mM potassium acetate/HCl buffers, pH 4.0–5.4; and 100 mM KH2PO4/NaOH buffers, pH 6.0–8.0. The reaction was stopped by the addition of 100 μl of 0.2 M sodium phosphate buffer, pH 8.0, and 100 μl of the reaction mixture was analyzed by HPLC in the same manner as above.

**Treatment with EDC and Glicynamide**—The enzyme (10 μM) was allowed to react with 40 mM EDC and 0.1 M glicynamide in 10 μl of 0.2 M MES buffer, pH 5.5, at 30 °C for 30 min to modify carboxyl groups. The reaction was stopped by the addition of 10 μl of 1 M sodium acetate, and the remaining activity was determined at pH 2.3. The reaction was also performed in the same manner in the presence of 100 μM propeptide except that the enzyme and the propeptide were mixed and incubated at pH 4.0 and 4 °C for 10 min before the reaction.

**Differential Scanning Calorimetric Analysis**—The sample solutions were prepared to contain the enzyme (0.5 mg/ml, 67 μM) and each synthetic peptide (0–580 μM) in 50 mM sodium acetate buffer, pH 5.6, containing 0.2 M sodium chloride and filtered and then degassed before use. The pH of 5.6 was chosen because the thermal unfolding of the enzyme was known to be quantitatively reversible at pH 5–6 (38). Differential scanning calorimetric (DSC) measurements were performed in the presence and absence of the propeptide or its fragments using a MicroCal MC-2 microcalorimeter (MicroCal Inc.) with a Haake F3 temperature-controlled water bath. The cells were placed under 0.15 megapascals of dry nitrogen during the scan. Denaturation curves were recorded from 10 to 100 °C at a scanning rate of 1 °C/min. Data analysis was performed using the software Origin for DSC version 2.9 (MicroCal Inc.). Similar experiments were also performed with porcine pepsin and rhizopuspepsin for comparison.

**RESULTS**

**Inhibition of AGP by the Propeptide**—Under the assay conditions used, substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met(NH2)) was specifically cleaved at the Phe-Gly bond to give two fragments, Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe and Gly-Leu-Met(NH2), and the activity could be estimated from the amount of the octapeptide generated. The Km and Vmax values of the enzyme toward the substrate were determined from the Lineweaver-Burk plot to be 73 μM and 6.9 pmol·s−1, respectively. The activity toward substance P was found to be strongly inhibited by the propeptide. Fig. 1 shows a Dixon plot for the inhibition of the enzyme by the propeptide. The results indicate that the propeptide inhibits the enzyme competitively with a Ki value of 0.027 μM. The propeptide had no inhibitory effect on porcine pepsin when examined under similar conditions (data not shown).

**Inhibition of AGP by Truncated Propeptides**—Table I summarizes the Ki values of peptides in which the N- and/or C-terminal region of the propeptide was truncated. Shortening of the propeptide led to a decrease in the inhibitory activity to various extents. Among the truncated propeptides examined, the central 20-residue peptide Glu12–Lys31 (peptide 6; Km = 0.037 μM) and Ala1–Lys31 (peptide 2; K = 0.045 μM) showed strong inhibition similar to the propeptide (peptide 1), and Glu12–Asn41 (peptide 5; K = 0.14 μM) was somewhat less inhibitory. On the other hand, the N-terminal half (peptide 3, Ala1–Ser21; K = 1.5 μM) and the C-terminal half (peptide 8, Asn22–Asn41; 140 μM) of the propeptide showed much weaker inhibition, as did the N-terminal 12-residue peptide Ala1–Glu12 (peptide 4; K = 53 μM) and the C-terminal 10-residue peptide Glu12–Asn41 (peptide 10; K = 370 μM) as well as the N-terminal half (peptide 7, Glu12–Ser21; K = 110 μM) and the C-terminal half (peptide 9, Pro23–Lys31; K = 64 μM) of the central 20-residue peptide were without significant inhibitory activity.

**Inhibition of AGP by Truncated Peptides of Glu12–Lys31**—Table II summarizes the results obtained with a series of peptides where the N- and/or C-terminal region of the central 20-residue peptide Glu12–Lys31 (peptide 6) was further truncated. After removal of the N-terminal 6 residues, the resulting peptide Lys18–Lys31 (peptide 16) retained a fairly strong inhibitory effect, whereas the other peptides were without significant inhibitory activity.
The inhibitory activity (K<sub>i</sub> = 0.13 μM), and further removal of Lys<sup>18</sup> resulted in an ∼5-fold reduction in the inhibitory activity (see peptide 17, Arg<sup>19</sup>–Lys<sup>31</sup>). However, more drastic changes in the inhibitory activity were observed upon removal of the subsequent two residues. Removal of Arg<sup>19</sup> resulted in an ∼60-fold reduced inhibition (see peptide 18, His<sup>20</sup>–Lys<sup>31</sup>; K<sub>i</sub> = 43 μM), and furthermore the removal of His<sup>20</sup> abolished the inhibitory activity almost completely (see peptide 19, Ser<sup>21</sup>–Lys<sup>31</sup>). On the other hand, when the C-terminal 5 residues were removed from Lys<sup>18</sup>–Lys<sup>31</sup> (peptide 16), the resulting 9-residue peptide Lys<sup>18</sup>–Ile<sup>26</sup> (peptide 21) retained a fairly strong inhibitory activity (K<sub>i</sub> = 0.27 μM), and Lys<sup>18</sup>–Tyr<sup>25</sup> (peptide 22), which without Ile<sup>26</sup> was still fairly inhibitory. The tetrapeptide Arg<sup>19</sup>–Asn<sup>22</sup> (peptide 23), however, showed no inhibition.

**Inhibition of AGP by Ala Scan-substituted Peptides of Glu<sup>12</sup>–Lys<sup>31**—To pinpoint the region that contributes most to the inhibitory activity, a further study was performed with a series of Ala scan-substituted peptides of the central 20-residue peptide Glu<sup>12</sup>–Lys<sup>31</sup>, and the results are summarized in Table III. The most striking decrease in the inhibitory activity was observed when His<sup>20</sup> or Arg<sup>19</sup> was replaced with alanine; the inhibitory activity of the parent peptide Glu<sup>12</sup>–Lys<sup>31</sup> (peptide 6; K<sub>i</sub> = 0.037 μM) was reduced by 35-fold (peptide 27; K<sub>i</sub> = 1.3 μM) or 23-fold (peptide 26; K<sub>i</sub> = 0.83 μM). In addition, the inhibitory activity was reduced significantly (∼6–9-fold) by Ala substitution of Asn<sup>22</sup> (peptide 29), Ser<sup>21</sup> (peptide 28) or Pro<sup>23</sup> (peptide 30). The substitution of Pro<sup>24</sup> (peptide 31), Lys<sup>18</sup> (peptide 25), or Arg<sup>14</sup> (peptide 24) also reduced the inhibitory activity, but to a lesser extent (∼3–4-fold). On the other hand, the substitution of Tyr<sup>25</sup> (peptide 32) or Ile<sup>26</sup> (peptide 33) was without much effect. Multiple Ala substitutions at relevant residues resulted in further loss of inhibitory activity; especially the double substitution of Arg<sup>19</sup> and His<sup>20</sup> (peptide 34), Ser<sup>21</sup> and Asn<sup>22</sup> (peptide 35), or Pro<sup>23</sup> and Pro<sup>24</sup> (peptide 36) was effective to reduce the activity by ∼100–400-fold, and the simultaneous substitutions of Arg<sup>19</sup>, His<sup>20</sup>, Pro<sup>23</sup>, and Pro<sup>24</sup> (peptide 38) abolished the activity almost completely.

**Hydrolysis of Lys<sup>18</sup>–Tyr<sup>25</sup> (P23A/P24A) by AGP.—**All the peptides so far examined including the complete propeptide appeared to be resistant to the action of the enzyme at pH 4.0 as judged from the HPLC patterns obtained in the inhibition assay. Indeed, no hydrolysis occurred when the propeptide (Ala<sup>1</sup>–Asn<sup>41</sup>), Glu<sup>12</sup>–Lys<sup>31</sup> (peptide 6), Lys<sup>18</sup>–Tyr<sup>25</sup> (peptide 22), and a series of Ala scan-substituted Lys<sup>18</sup>–Tyr<sup>25</sup> were incubated with the enzyme for a longer period. Under the same conditions, however, the 8-residue peptide Lys<sup>18</sup>-Arg-His-Ser-Asn-Ala-Ala-Tyr, which had been derived from the central 8-residue peptide Lys<sup>18</sup>–Tyr<sup>25</sup> (peptide 22) by replacing the Pro-Pro sequence with an Ala-Ala sequence, was found to be hydrolyzed by the enzyme at pH 4.0 in the His-Ser bond (extent of hydrolysis: ∼20% in 3 h under the conditions used) (data not shown).

**pH Dependence of the Inhibition of AGP by the Propeptide—**Fig. 2 shows the pH dependence of the hydrolysis rates of substance P in the presence and absence of the propeptide. In the absence of the propeptide, a rather broad pH dependence curve was obtained with a maximum at pH around 2 and the half-maximal value at pH 5.6, and almost no activity was observed above pH 7.0. On the other hand, in the presence of a 3.8-fold molar excess of the propeptide, a remarkable inhibition was observed in the pH range of 3.5–6.0 with a maximum inhibition at pH 5.0. The relative extents of inhibition as a
The activity was determined by incubating a mixture of 40 nM tide. The activity was taken as 100% (relative activity). The maximum activity obtained at pH 2.0 in the absence of the propeptide was relative activity in the presence of the propeptide; extents of inhibition (%). The maximum activity obtained at pH 2.0 in the absence of the propeptide was taken as 100% (relative activity).

Protective Effect of the Propeptide on the Inactivation of AGP by EDC/Glycinamide—The results of reaction with EDC/glycinamide in the presence and absence of the complete propeptide is shown in Fig. 3. When the enzyme was treated with EDC and glycinamide at pH 5.5 and 30 °C for 30 min under the specified conditions, it was markedly inactivated; 14% of the original activity remained as assayed at pH 2.3 (second bar). When the enzyme was preincubated with a 10-fold molar excess of the propeptide at pH 4.0 and then assayed at pH 2.3, it showed 53% of the original activity (third bar). On the other hand, when this enzyme/propeptide mixture was treated with EDC and glycinamide at pH 5.5 and 30 °C for 30 min and then assayed at pH 2.3, 60% of the activity before the treatment was retained (fourth bar).

Thermal Stabilization of AGP by the Propeptide—The results of DSC of the enzyme at pH 5.6 are shown in Fig. 4. The pH of 5.6 was chosen because the thermal unfolding of the enzyme was known to be quantitatively reversible at pH 5–6 (38). Fig. 4A shows denaturation curves for the enzyme at different molar ratios of the enzyme and the complete propeptide. It is notable that the denaturation took place biphasically. The T\textsubscript{m} value of the free enzyme was 52.9 °C, and the corresponding ΔH\textsubscript{cal} value was calculated to be 562 kJ/mol. On the other hand, when the molar ratio of the propeptide over the enzyme was 2.5, the T\textsubscript{m} value of 71.7 °C was obtained, where the ΔH\textsubscript{cal} value was calculated to be 727 kJ/mol. Thus, under the conditions used, the T\textsubscript{m} value of the enzyme-propeptide complex was by 18.8 °C higher than that of the free enzyme. Under the same conditions, the DSC curve was flat, and no peak was seen with the propeptide alone (data not shown). The correlation of the ΔH\textsubscript{cal} value to the molar ratio of the propeptide over the enzyme was biphasic as can be seen from Fig. 4B, and an inflection point was obtained at the propeptide/enzyme molar ratio of ~0.9. For comparison, porcine pepsin and rhizopuspepsin were submitted to the DSC analysis, which gave T\textsubscript{m} values of 57.3 and 67.3 °C, respectively. These T\textsubscript{m} values, however, did not change when they were incubated with the propeptide under the same conditions (data not shown).

Thermal Stabilization of AGP by Truncated Propeptides and Ala Scan-substituted Peptides of Lys\textsuperscript{18}–Tyr\textsuperscript{25}—The denaturation curves of the enzyme in the presence and absence of some typical truncated propeptides and Ala scan-substituted peptides of Lys\textsuperscript{18}–Tyr\textsuperscript{25} are shown in Fig. 5. Among the four 20-residue propeptide fragments, Glu\textsuperscript{12}–Lys\textsuperscript{31} (peptide 6) was

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**Table III**

| No. | Spanning/mutant | Amino acid sequence | K\textsubscript{i} μM |
|-----|-----------------|---------------------|------------------|
| 6   | Glu\textsuperscript{12}–Lys\textsuperscript{31} | EARAAGKRHSNPPYYPGSDK | 0.037 |
| 24  | R14A            | EARAAGKRHSNPPYYPGSDK | 0.12  |
| 25  | K18A            | EARAAGKRHSNPPYYPGSDK | 0.13  |
| 26  | R19A            | EARAAGKRHSNPPYYPGSDK | 0.83  |
| 27  | H20A            | EARAAGKRHSNPPYYPGSDK | 1.3   |
| 28  | S21A            | EARAAGKRHSNPPYYPGSDK | 0.29  |
| 29  | N22A            | EARAAGKRHSNPPYYPGSDK | 0.32  |
| 30  | P23A            | EARAAGKRHSNPPYYPGSDK | 0.22  |
| 31  | P23A/P24A       | EARAAGKRHSNPPYYPGSDK | 0.13  |
| 32  | Y25A            | EARAAGKRHSNPPYYPGSDK | 0.051 |
| 33  | I26A            | EARAAGKRHSNPPYYPGSDK | 0.059 |
| 34  | R19A/H20A       | EARAAGKRHSNPPYYPGSDK | 11    |
| 35  | S21A/N22A       | EARAAGKRHSNPPYYPGSDK | 17    |
| 36  | P23A/P24A       | EARAAGKRHSNPPYYPGSDK | 5.1   |
| 37  | P23A/P24A/P27A  | EARAAGKRHSNPPYYPGSDK | 3.7   |
| 38  | R19A/H20A/P23A/P24A | EARAAGKRHSNPPYYPGSDK | 200  |
Asn22–Asn41 (peptide 8) had little effect. It is notable that both of Lys18–Tyr25 examined had practically no stabilizing effect. On the other hand, the six Ala scan-substituted peptides retained some stabilizing potencies. The propeptide, whereas Ala1–Ser21 showed a monophasic transition. The residues with $pK_a$ values for these peptides are summarized in Tables IV and V.

**DISCUSSION**

As described above, the synthetic 41-residue propeptide of AGP was shown to be a potent competitive inhibitor of the enzyme, suggesting the possibility that the propeptide or part of it may bind directly to the active site of the enzyme. This supposition is consistent with the fact that the enzyme was significantly protected by the propeptide from inactivation with EDC/glycinamide, which is thought to involve the modification of the carboxyl group of the catalytic glutamic acid residue. Although the enzyme was not completely protected by the propeptide, this may be due to partial inactivation of the enzyme by nonspecific modification of other carboxyl groups. The inhibitory effects of the full-length propeptides on the corresponding mature enzymes have been reported for a number of peptidases; in many cases, they seem to show a competitive type inhibition with varying inhibition constants ($K_i = 0.1–2000 \text{ nM}$). Therefore, the type of inhibition of AGP by its propeptide is similar to that for many other peptidases, and the inhibitory potency of the propeptide is reasonably high as compared with several other peptidases.

The results obtained with various shorter propeptide fragments (Table I) indicated that the central 20-residue segment Glu12–Lys31 (peptide 6) of the propeptide contributes mainly to the inhibition, although other regions may additionally contribute to some extents to the inhibition. The results with several N- and/or C-terminally truncated peptides of Glu12–Lys31 (Table II) further restricted the major inhibitory site to Lys18–Tyr25 (peptide 22). On the other hand, the results obtained with the Ala scan-substituted peptides of Glu12–Lys31 (Table III) indicated that His20 and Arg19 are the most critical residues contributing to the inhibition. In addition, Asn22, Ser21, Pro23, and Pro24 were shown to be also important, and their contribution to the inhibition decreased in this order; these residues are thought to contribute cooperatively with His20 and Arg19 to the inhibition through interactions with several sites of the enzyme. Lys18 and Arg14 contributed to lesser extents to the inhibition. These results will be useful for developing effective peptide-based inhibitors for the enzyme.

On the other hand, Lys18–Tyr25 (peptide 22) (P23A/P24A), Lys-Arg-His-Ser-Asn-Ala-Ala-Tyr, was found to be cleaved at the His-Ser bond by the enzyme, suggesting that His20 occupies the P1 position of this substrate peptide, thus interacting with the S1 site in the enzyme. The cleavage after His is consistent with the known cleavage specificity of AGP (35, 39, 40). Taken together, it seems most likely that the propeptide and its inhibitory fragments bind to the enzyme active site as substrate analogs to inhibit the enzymatic activity. They presumably bind to the enzyme in the same manner as they do when they are covalently bound to the enzyme moiety in the proenzyme. The positively charged side chains of Arg19 and His20 are supposed to interact specifically with some of the acidic residues at or near the active site of the enzyme. It is tempting to assume that the imidazolium group of His20 may interact directly with the carboxylate group of the catalytic glutamic acid by electrostatic interaction. The residues with $pK_a$ values in the ranges of 4–5 and 5–6, which were suggested to be involved in the inhibition, may correspond to these acidic residues in the enzyme. It may be also possible that the latter $pK_a$ values reflect the deprotonation of the side chain of His20. It is interesting that the double substitution, but not the single substitution, of the two prolines in Lys18–Tyr25 with alanine changed the inhibitory potency of the propeptide. Presumably, the mode of binding of the peptide would be changed to a productive one through a subtle change in the peptide/protein interaction induced by these substitutions, whereas the inhibitor with the two prolines should bind to the enzyme in a nonproductive mode. Interestingly, the homologous enzyme scytalidoglutamic peptidase was reported to cleave angiotensin II at the His-Pro bond; part of the product, Tyr-Val-His, remains attached to the active site with the His-Ser bond by the enzyme, suggesting that His20 occupies the P1 position of this substrate peptide, thus interacting with the S1 site in the enzyme. The cleavage after His is consistent with the known cleavage specificity of AGP (35, 39, 40). Taken together, it seems most likely that the propeptide and its inhibitory fragments bind to the enzyme active site as substrate analogs to inhibit the enzymatic activity. They presumably bind to the enzyme in the same manner as they do when they are covalently bound to the enzyme moiety in the proenzyme. The positively charged side chains of Arg19 and His20 are supposed to interact specifically with some of the acidic residues at or near the active site of the enzyme. It is tempting to assume that the imidazolium group of His20 may interact directly with the carboxylate group of the catalytic glutamic acid by electrostatic interaction. The residues with $pK_a$ values in the ranges of 4–5 and 5–6, which were suggested to be involved in the inhibition, may correspond to these acidic residues in the enzyme. It may be also possible that the latter $pK_a$ values reflect the deprotonation of the side chain of His20. It is interesting that the double substitution, but not the single substitution, of the two prolines in Lys18–Tyr25 with alanine changed the inhibitory potency of the propeptide. Presumably, the mode of binding of the peptide would be changed to a productive one through a subtle change in the peptide/protein interaction induced by these substitutions, whereas the inhibitor with the two prolines should bind to the enzyme in a nonproductive mode. Interestingly, the homologous enzyme scytalidoglutamic peptidase was reported to cleave angiotensin II at the His-Pro bond; part of the product, Tyr-Val-His, remains attached to the active site with the P1 His in the S1 site (28).

Similar studies to identify critical sequences and/or residues for inhibition in the propeptide have been reported for only a few peptidases, such as pepsin (41), cathepsin B (14, 42), and proprotein convertases (16, 23–25). Porcine pepsin was re-
FIG. 5. Thermal denaturation curves of AGP in the presence of varying amounts of truncated propeptides and their Ala mutants. The conditions for DSC measurements were the same as in Fig. 4 except that higher amounts of peptide were also used in some cases. A, Ala1–Ser21 (peptide 3); B, Asn22–Asn41 (peptide 8); C, Glu12–Lys31 (peptide 6); D, Lys18–Tyr25 (peptide 22); E, Lys18–Tyr25 (R19A) (peptide 39); F, Lys18–Tyr25 (H20A) (peptide 40); G, Lys18–Tyr25 (S21A) (peptide 41); H, Lys18–Tyr25 (N22A) (peptide 42); I, Lys18–Tyr25 (P23A) (peptide 43); J, Lys18–Tyr25 (P24A) (peptide 44). The amounts (nmol) of peptide used are shown.
The enzyme (67 nm) was mixed with a 170 nm concentration of each peptide. The denaturation curves in the presence of the propeptide and its truncated peptides showed biphasic transition (Fig. 5, C and D), consistent with the specific binding of these peptides to the enzyme. On the other hand, the denaturation curves in the presence of Ala\(^{1-}-\)Ser\(^{31}\), representing the N-terminal half of the propeptide, showed a monophasic transition (Fig. 5A), suggesting a nonspecific binding of the peptide to the enzyme; the basic amino acids in this peptide, including those in the N-terminal region, are assumed to mainly contribute to this nonspecific binding.

The propeptide showed no stabilizing effect on thermal denaturation of the aspartic peptidase porcine pepsin and rhizopuspepsin, indicating its specific binding to AGP. This is consistent with the fact that porcine pepsin was not inhibited by the propeptide of AGP. So far, the effect of the propeptide and its fragments on the stability of the mature enzyme has scarcely been investigated for other peptidases or enzymes. In the present study, the sequence most important for inhibition of the mature enzyme was shown to be essentially the same as that important for its thermal stabilization. This is the first time that such a short specific segment and residues therein were shown to be critical to both enzyme inhibition and stabilization in the propeptide of a peptidase. It would be interesting to study this relationship on other enzymes, since the sequences important for inhibition and stabilization should not necessarily be the same in other enzymes. In the pro-AGP, the propeptide is supposed to take a definite conformation when bound to the enzyme, although the propeptide alone had no definite secondary structure as examined by circular dichroism spectroscopy (data not shown). Thus, further elaborate studies including x-ray analysis of the three-dimensional structure of the propeptide (or its inhibitory fragment)-enzyme complex as well as of the proenzyme itself will be necessary to clarify in more detail the molecular mechanism of the inhibition and stabilization of AGP by its propeptide.

**Acknowledgments**—We are grateful to Dr. Kaori Hiraga (Tokyo University of Pharmacy and Life Science) for technical advice in the DSC measurements and to Drs. Akihiko Yamagishi and Heizaburo Shindo (Tokyo University of Pharmacy and Life Science) for helpful discussions. We also thank Drs. Toshihuki Kono and Yuichi Sato (Bioscience Laboratories, Meiji Seika Co.) for the generous supply of Proctase and help in preparation of AGP.

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