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Folding and Stability of the Z and S_{iiyama} Genetic Variants of Human α_1-Antitrypsin*

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Z (Glu^{342} → Lys) and S_{iiyama} (Ser^{53} → Phe) genetic variations of human α_1-antitrypsin (α_1-AT) cause a secretion blockage in the hepatocytes, leading to α_1-AT deficiency in the plasma. Using *in vitro* folding analysis, we have shown previously that these mutations interfere with the proper folding of polypeptides. To understand the fundamental cause for the secretion defect of the Z and S_{iiyama} variants of α_1-AT, we investigated *in vivo* folding and stability of these variant α_1-AT using the secretion system of yeast *Saccharomyces cerevisiae*. Various thermostable mutations suppressing the folding block of the Z variant *in vitro* corrected the secretion defect as well as the intracellular degradation in the yeast secretion system. Significantly, the extent of suppression in the secretion defect of Z protein was proportional to the extent of suppression in the folding defect, assuring that the *in vivo* defect associated with the Z variant is primarily derived from the folding block. In contrast, the folding and secretion efficiency of S_{iiyama} was not much improved by the same mutations. In addition, none of the rarely secreted S_{iiyama} α_1-AT carrying the stabilizing mutations for the wild type and Z variant were active. It appears that the major defect in S_{iiyama} variant is the loss of stability in contrast to the kinetic block of folding in the Z variant.

Human α_1-antitrypsin (α_1-AT)[1] is a member of the serine protease inhibitor family, which has a tertiary structure composed of three β-sheets and several α-helices (1). α_1-AT is synthesized as a glycoprotein of 394 amino acids in the liver and is secreted into the blood. Its major physiological function is to protect the lungs from excessive elastase activity, and thus its deficiency causes the lung disease emphysema (2, 3). Some genetic variations of α_1-AT, such as Z (Glu^{342} → Lys), S_{iiyama} (Ser^{53} → Phe), and M_{malton} (Phe^{52} → deleted), cause α_1-AT deficiency in plasma by blocking the secretion of α_1-AT (4). The secretion blockage of these variant α_1-AT appears to be due to the aggregation of the proteins (5–7). Most of the newly synthesized variant α_1-AT accumulate as aggregates and are retained in the endoplasmic reticulum (ER) of the hepatocytes, which are eventually degraded. Only a small fraction of the protein that escapes degradation is deposited as insoluble aggregates within the hepatic ER (8), resulting in liver diseases.

The structural basis of aggregation of the variant α_1-AT has been suggested to be the loop-sheet polymerization in which a partial opening of the central β-sheet, the A β-sheet, accompanies the insertion of the reactive center loop of another molecule (4). The loop-sheet polymerization appears to be an important contributing factor for the secretion blockage of the variant α_1-AT. This implication is based on the finding that the secretion blockage of the Z or S_{iiyama} was efficiently suppressed in the *Xenopus* oocyte system either by a thermostable mutation (Phe^{51} → Leu: F51L) that enhanced the closure of A β-sheet or by mutations that decreased the loop mobility (9). Various other biochemical and pathological data for the above mutations and other serpin mutations (10) strongly support that the loop-sheet polymerization is a structural basis of *in vivo* aggregation. However, the mechanism by which each individual mutant α_1-AT tends to polymerize and thus cause secretion blockage remains unclear. One possible mechanism for the polymerization of the mutant α_1-AT is that the kinetic trap during folding results in the accumulation of intermediates with a high tendency to polymerize. Another possible mechanism is that the stability of the already folded structure is lost, resulting in a conformational change needed for polymerization.

To test these possibilities here, we systematically investigated the folding, stability, and the secretion of the Z and S_{iiyama} variants of α_1-AT, using *in vitro* folding assay and yeast secretion system. In order to assess the degree of individual defects, we examined their suppression by various thermostable mutations of α_1-AT which were identified recently (11). The results in the present study demonstrate that there is a direct correlation between the suppression of folding block and the suppression of secretion block of the Z variant. In contrast, the defect of S_{iiyama} is much more difficult to rescue than the Z type defect, which is presumably due to another kind of defect, *i.e.* stability loss.

MATERIALS AND METHODS

Strains and Plasmids—The recombinant yeast strain expressing the wild type human α_1-AT protein has been described previously (12). Briefly, the recombinant *Saccharomyces cerevisiae* Y2805 (*MATα pep::HIS3 prb1-Δ1.6R can1 his3–200 ura3–52*) harbors the α_1-AT expression vector, pYInu-AT, that contains the cDNA of human α_1-AT fused in frame with the inulinase signal sequence downstream of the *GAL10* promoter. The yeast expression vectors for variant α_1-AT proteins were constructed by exchanging the 1.1-kilobase pair *Bcl*I/*Sal*I fragment containing the coding sequence of α_1-AT (starting at 17th codon of mature α_1-AT) in pYInu-AT with the corresponding fragment derived from the pF(BGL)AT plasmids that were used in the *in vitro* transcription/translation.

In Vitro Folding Assay—To express the mutant forms of α_1-AT protein *in vitro*, the cDNA fragment coding for the wild type α_1-AT in the plasmid pF(BGL)AT (13) was replaced with the corresponding cDNA fragment encoding the thermostable mutants derived from the mutagenized plasmid pFEAT30 (11). The Z and the S_{iiyama} mutation were introduced into the cDNA encoding the thermostable mutations by oligonucleotide-directed mutagenesis (14). *In vitro* transcription and

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[1] The abbreviations used are: α_1-AT, α_1-antitrypsin; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; endo H, endoglycosidase H; TUG gel, transverse urea gradient gel.

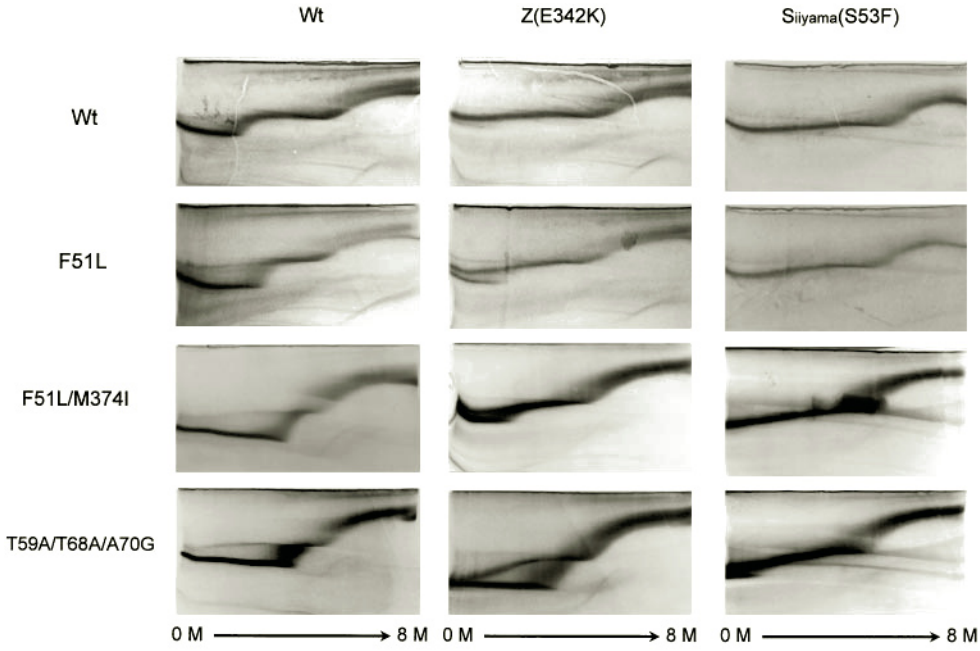

FIG. 1. **Suppression of the folding defect of Z and S_{iiyama} variants by the thermostable mutations.** The α_1-AT proteins carrying the indicated mutations were synthesized in the presence of [^{35}S]methionine for 1 h by the *in vitro* translation system. The folding state of the *in vitro* translation product was then analyzed on the transverse urea gradient gel, in which a gradient of 0–8 M urea was applied from the left to the right. The protein bands were visualized by autoradiography.

translation of the cloned gene were carried out and the translation products were analyzed by transverse urea gradient (TUG) gel as previously described (13).

Biosynthetic Labeling and Immunoprecipitation—The recombinant yeast cells were grown in methionine-labeling medium containing 2% galactose, 0.67% yeast nitrogen base without amino acids, adenine, and amino acids except methionine until the culture reached the mid-exponential growth phase. After harvesting, the cells were resuspended and incubated in the same medium containing [^{35}S]methionine (Amersham Corp.). For pulse-chase analysis, the cells were pelleted and resuspended in the labeling medium containing 0.2 mg/ml nonradioactive methionine. The cultures were removed at various times and fractionated into cell pellet and culture supernatant. The cell pellets were resuspended in buffer A (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Na-HEPES, pH 7.5, 0.5 mg/ml bovine serum albumin, 1 mM phenylmethysulfonyl fluoride, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM dithiothreitol) and disrupted by vortexing with 0.1 ml of glass beads. The obtained cell lysates (intracellular fraction) and the culture supernatants (extracellular fraction) were analyzed by immunoprecipitation with the polyclonal antibody against human α_1-AT (Sigma) according to the procedure described previously (15). The immunoprecipitates were analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE). Gels were fixed, amplified, and fluorographed with EN^{3}HANCE (DuPont). For endoglycosidase H (endo H) treatment, immunoprecipitates were suspended in 54 μl of 1% SDS, 10% glycerol, 1% 2-mercaptoethanol and incubated for 10 min at 95 °C. After cooling, 6 μl of 0.5 M sodium citrate, pH 5.5, and 100 unit endo H were added. The samples were incubated overnight at 37 °C and analyzed by 10% SDS-PAGE.

Western Blot Analysis—Total yeast cell lysates (intracellular fraction) were obtained by vortexing cell pellets resuspended in the Laemmli sample buffer (2% SDS, 10% glycerol, 2% 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, 0.01% bromphenol blue) together with glass beads. The culture supernatant (extracellular fraction) was concentrated 20-fold by ultrafiltration (Amicon, YM30 membrane). Western blot analysis of the samples was carried with the polyclonal antibody raised against human α_1-AT and peroxidase-conjugated anti-rabbit IgG goat antibody (Sigma). The color development of peroxidase reaction was carried out using 3,3′,5,5′-tetramethylbenzidene stabilized substrate (Promega).

Assay for α_1-AT Activity—The biological activity of α_1-AT in the culture supernatant was measured for elastase inhibitory activity according to the method described previously (12).

RESULTS

Folding and Stability of the Z and S_{iiyama} Variants of α_1-AT—To investigate the effects of various mutations of α_1-AT on the folding, the conformational states of the α_1-AT variants translated *in vitro* were analyzed by transverse urea gradient gel electrophoresis (Fig. 1). As observed previously (13), the folding of the nascent Z polypeptides was blocked at an intermediate state, but the presence of the F51L mutation (T1) converted some (40–50%) of the Z-type polypeptides into the native state. When the Z mutation was combined with the double mutation (F51L/M374I: T2), or the triple mutation (T59A/T68A/A70G: T3), that increased the stability of α_1-AT more than F51L (T1) (10), a greater fraction (70–90% *versus* 40–50%) of the polypeptides folded into the native form (Fig. 1, *middle column*). The results suggest that the extent of suppression in the folding defect is related to the extent of stability increase of the combining mutations. The effects of the thermostable mutations on the conformational stability of the native Z protein do not appear to be additive in all cases, as revealed by the transition midpoint in the TUG gel (Fig. 1). The stability increase conferred by T2 on urea denaturation was similar to that of T3 (Fig. 1, *left column*) with the wild type background. However, the stability of the native Z/T2 was much lower than that of Z/T3 (*middle column*), and the fraction of the Z polypeptides restored to the native state by T2 was less than that by T3 mutation (70 *versus* 90%). The stability of the native Z/T3 was almost the same as that of T3, whereas the stability of the native Z/T1 and Z/T2 was much lower than that of T1 and T2, respectively.

The *in vitro* translation products of α_1-AT carrying S_{iiyama} also could not fold to the native state (Fig. 1, *right column*). In contrast to the Z type folding block, which was suppressed by all three thermostable mutations (T1–T3), the defect of S_{iiyama} was suppressed only by T3. About 20% of the S_{iiyama} polypeptides were rescued to the native state by T3. The stability of the native S_{iiyama}/T3 was much lower than that of T3, and even lower than that of the wild type.

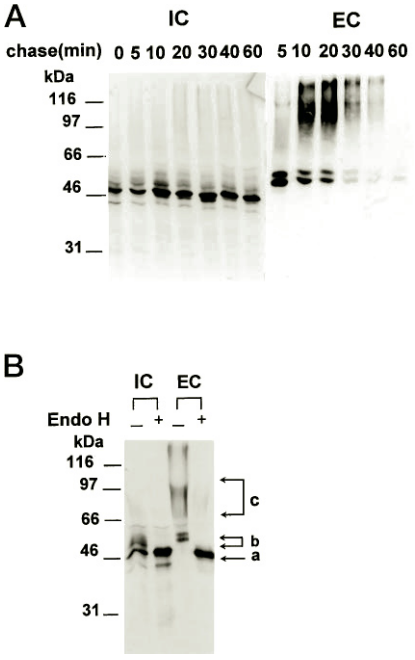

FIG. 2. **Expression and secretion of human α_1-AT proteins from *S. cerevisiae*.** *A*, kinetics of secretion of the wild type α_1-AT in yeast. The yeast transformants expressing human α_1-AT fused to the inulinase signal sequence were pulse-labeled with [^{35}S]methionine for 2 min and then chased in the presence of excess unlabeled methionine for the indicated time. At the end of each interval, intracellular lysate (*IC*) and extracellular medium (*EC*) were subjected to immunoprecipitation with polyclonal antibody raised against human α_1-AT. The immunoprecipitates were fractionated on 10% SDS-PAGE, followed by flourography. Molecular mass markers are indicated in kilodaltons on the *left*. *B*, *N*-linked glycosylated forms of human α_1-AT from yeast. The immunoprecipitates from the intracellular fraction (*IC*) and extracellular fraction (*EC*) from *A* were incubated without (−) or with (+) endo H overnight at 37 °C and analyzed by 10% SDS-PAGE flourography. *a*, unglycosylated form of α_1-AT; *b*, core-glycosylated form of α_1-AT; and *c*, hyperglycosylated form of α_1-AT.

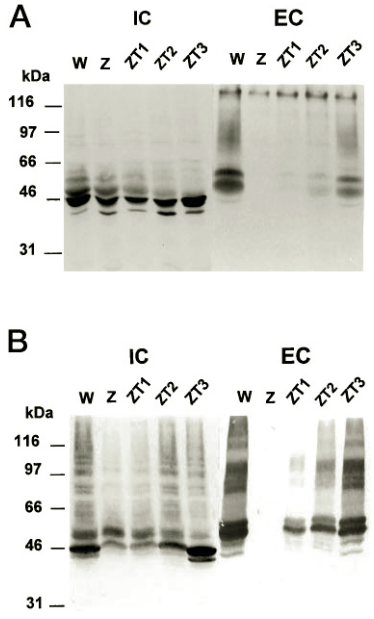

FIG. 3. **Effect of the thermostable mutations on the secretion block of Z variant protein in yeast.** *A*, pulse labeling analysis of α_1-AT secretion. The yeast transformants expressing the wild type α_1-AT (*W*), the Z variant (*Z*), the Z variants combined with F51L mutation (*ZT1*), F51L/M374I mutation (*ZT2*), or T59A/T68A/A70G mutation (*ZT3*) were pulse-labeled for 10 min with [^{35}S]methionine. Radiolabeled proteins were immunoprecipitated from intracellular lysates (*IC*) and extracellular medium (*EC*) and then analyzed by SDS-PAGE fluorography. *B*, Western blot analysis of α_1-AT secretion. To detect the steady-state level of α_1-AT proteins, the yeast transformants were cultured for 2 days in the YP media containing 1% glucose and 1% galactose. The total intracellular extract (*IC*) and the concentrated extracellular medium (*EC*) were fractionated on 10% SDS-PAGE and analyzed by the polyclonal α_1-AT antibody.

Secretion of Human α_1-AT in Yeast System—As a model system to assess the effects of various mutations on the *in vivo* folding and secretion of α_1-AT protein, a yeast expression system (12) with high-level secretion of biologically active human α_1-AT into extracellular culture medium was utilized. To examine the kinetics of secretion of α_1-AT in the recombinant yeast, the cells expressing the wild type human α_1-AT were analyzed by pulse-chase and immunoprecipitation experiments. The immunoprecipitation of the obtained culture supernatants (Fig. 2*A, EC*) showed that significant amounts of the core-glycosylated α_1-AT were already secreted outside the yeast cells within 5-min chase following 2-min pulse labeling with [^{35}S]methionine. The extensively hyperglycosylated forms (above 54 kDa) began to show up later, which reflects a longer transit time for such forms in the secretion pathway due to the additional modification of α_1-AT protein. The level of secreted α_1-AT increased up to 20 min of chase but after that the level decreased, probably due to the degradation by the action of yeast extracellular proteases. Analysis of the soluble intracellular fraction (Fig. 2*A, IC*) showed that a major polypeptide of ~46 kDa remained stable throughout the chase period. The intracellularly retained α_1-AT of ~46 kDa appears to be the unglycosylated precursor form, since the endo H treatment did not affect the size at all, while the treatment converted all the heterogeneously glycosylated forms in the culture supernatant into a single form of ~46 kDa (Fig. 2*B*). Most of the unglycosylated form of α_1-AT was found in the cytosolic fraction of yeast and the size of the unglycosylated α_1-AT in yeast was slightly larger than that of α_1-AT expressed in *Escherichia coli* (data not shown). The results imply that some portions of the wild type α_1-AT synthesized in yeast were not translocated into the ER and remained stable in cytoplasm, which is likely due to high-level expression of the human α_1-AT protein in a heterologous host system. The results in Fig. 2, however, show that major portions of the human α_1-AT entered into the yeast ER and transited through the yeast secretion system very rapidly, with a similar transit time (less than 7 min) as those reported in other yeast secretory proteins (16).

Effect of Various Thermostable Mutations of α_1-AT on the Secretion Defect of Z and S_{iiyama}—If the folding defect is the direct cause of the secretion block associated with the Z variant, the suppression of the folding defect by the thermostable mutations (Fig. 1) should suppress the secretion blockage of Z variant observed *in vivo*. To compare the secretion rate of the variant forms of α_1-AT, the yeast transformants expressing α_1-AT were pulse-labeled for 10 min with [^{35}S]methionine and analyzed by immunoprecipitation. There was no detectable Z-type α_1-AT protein in the culture supernatant after the 10-min pulse (Fig. 3*A, EC, lane Z*). In combination with the thermostable mutations, however, some Z proteins were secreted into the culture media (Fig. 3*A, EC*). Ten minutes was chosen for the labeling time to avoid the degradation effect as much as possible. During the pulse, the cells expressing the wild type or the thermostable mutant proteins alone secreted similar amounts of α_1-AT proteins (data not shown). On the combination of Z mutation, the T1 substitution, which restored only a small part of the population of the Z polypeptide into the native state in the TUG gel (Fig. 1), released only a small fraction (10%) of the Z proteins from the secretion blockage (Fig. 3). With the T3, in which most of the Z polypeptides could fold into the native form, the secretion yield was enhanced up to 75% of

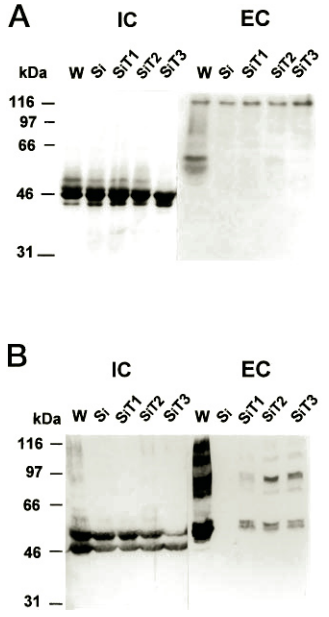

FIG. 4. **Effect of thermostable mutations on the secretion block of S_{iiyama} variant.** *A*, pulse labeling analysis of S_{iiyama} α_1-AT secretion. The yeast transformants expressing the wild type α_1-AT (W), the S_{iiyama} variant (*Si*), the S_{iiyama} variants combined with F51L mutation (*SiT1*), F51L/M374I mutation (*SiT2*), or T59A/T68A/A70G mutation (*SiT3*) were pulse-labeled 10 min with [^{35}S]methionine. Immunoprecipitates from intracellular lysates (*IC*) and extracellular medium (*EC*) were then analyzed by SDS-PAGE fluorography. *B,* Western blot analysis of S_{iiyama} α_1-AT secretion. The total intracellular extract (*IC*) and the concentrated extracellular medium (*EC*) were analyzed by the polyclonal α_1-AT antibody as described in the legend to Fig. 3.

the wild type protein. The T2 mutation improved the secretion of Z protein to an the intermediate extent (30%) as observed in the folding analysis. The results clearly show that the extent of suppression in the secretion was proportional to the extent of suppression in the folding of Z protein. The suppression of the secretion defect was also confirmed by Western blot analysis of the total cell lysates and the culture supernatant (Fig. 3*B*). There was no significant difference in the relative amounts of the secreted Z proteins, as measured either by pulse labeling (Fig. 2*B, EC*) or by Western blot (Fig. 3*B, EC*). The results indicate that the secreted Z proteins retain a substantial stability against degradation comparable with the wild type protein once they are secreted into the culture medium.

The secretion of S_{iiyama} variant was also blocked in the yeast secretion system (Fig. 4*A, EC*), and the secretion blockage was able to be suppressed slightly by all the three mutations. Compared with the suppression of the Z-type defect, however, the overall efficiency of the suppression of the S_{iiyama} type secretion was much lower. In Western blot (Fig. 4*B, EC*), the steady-state level of the secreted S_{iiyama} carrying the various mutations was 5~20%, while that of Z type was 10~75% (Fig. 3*B, EC*). The T2 and T3 were more efficient than T1 in suppressing the secretion defect of S_{iiyama}, but there was no difference between the suppression by T2 and that by T3.

To investigate that the secreted protein was correctly folded into the native form, the inhibitory activity of α_1-AT in the culture supernatant was measured by examining the ability to inhibit the activity of elastase (Table I). There is a strong correlation between the elastase inhibition activity in the culture supernatant and the amount of α_1-AT protein detected in Western blot of the Z proteins, supporting that the secreted Z proteins achieve the fully folded native form. The results clearly demonstrate that the increased secretion efficiency of the Z protein is directly correlated with the improved folding

TABLE I
Elastase inhibition activity of the secreted α_1-AT variants

The secretion level of α_1-AT was estimated by densitometry scanning of the fluorograms in Figs. 3*A* and 4*A*. The secretion yield was calculated by dividing the amount of α_1-AT in the medium by the total amount synthesized over the labeling period (cells + medium). The biological activity of α_1-AT secreted from yeast was measured by the elastase inhibition activity of the concentrated culture supernatant. The values of secretion yield and inhibition activity were represented relative to those of the wild type α_1-AT. Designations of the mutants are the same as in Figs. 3 and 4.

α_1-AT proteins	Secretion yield	Inhibition activity
	%	
W	100	100
ZT1	10	2
ZT2	25	17
ZT3	75	68
SiT1	7	0
SiT2	22	0
SiT3	19	0

efficiency. In the case of S_{iiyama} protein, however, the secreted proteins did not show any detectable inhibition activity, implying that the S_{iiyama} proteins, even if successfully secreted outside cells, were not in the active native conformation.

Effect of the Thermostable Mutations on the Intracellular Fate of Z and S_{iiyama} α_1-AT—In general the transport-impaired polypeptides inside cells are susceptible to degradation in the secretion pathway (17). We investigated the fate of the Z and S_{iiyama} α_1-AT that accumulated intracellularly in the yeast cells. The kinetics of the ER retention and intracellular degradation of various α_1-AT proteins were examined by the pulse-chase analyses. Fig. 5 displays two populations of the newly synthesized α_1-AT polypeptides with different destiny in the yeast cells: one is the nontranslocated cytoplasmic α_1-AT (*a*) and the other is the ER-translocated α_1-AT (*b*). In the case of the normal human α_1-AT (W), most of the core-glycosylated form of α_1-AT (*b*), which represents the population entering the secretion pathway, disappeared from the intracellular fraction within 30-min chase because of the secretion into extracellular medium. The unglycosylated form, representing the population that does not enter the secretion pathway, remained quite stable throughout the chase period. In contrast, the core glycosylated form of Z α_1-AT protein remained relatively longer in the intracellular fraction than the wild type form (Fig. 5*A*), reflecting the longer retention of the Z protein in the yeast ER as observed in the hepatic ER. The level of the retained Z α_1-AT decreased eventually during the chase. Since there was almost no detectable Z protien in the extracellular media (Fig. 3), the disappearance of core glycosylayed Z protein in the intracellular fraction is caused mainly by degradation rather than by secretion. The unglycosylated Z form, which presumably resided in cytoplasm, disappeared more rapidly.

On combination of the Z mutation with the thermostable mutations T1 and T2 (Fig. 5*A*), the amount of core-glycosylated form of α_1-AT (*b*) did not decrease as rapidly as that of the Z type during the chase, reflecting less degradation. The transit of α_1-AT from the ER to the Golgi appears to be still retarded for the Z/T1, because the intracellular transit of the glycosylated forms was longer than that of the wild type. The secretion process appeared to be facilitated by T2 and T3, as the lesser amount of glycosylated forms was detected at 60 min of chase. Interestingly, the amount of core-glycosylated Z/T3 α_1-AT appears to be smaller than that of the wild type, implying that Z/T3 appears to transit the ER more rapidly than the wild type. The unglycosylated forms (*a*) of Z α_1-AT carrying the thermostable mutations were also protected from degradation (Fig. 5*A*) in proportion to the degree of the suppression in the folding

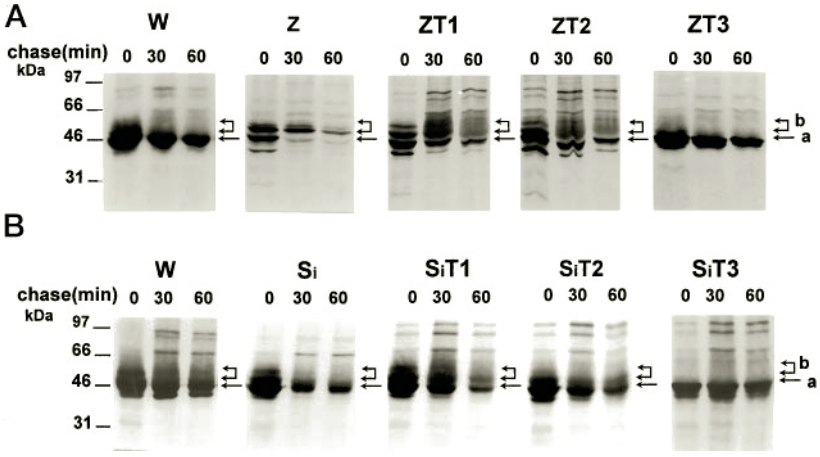

FIG. 5. **Suppression of the intracellular degradation of Z and S_{iiyama} variants by the thermostable mutations.** *A*, the yeast transformants expressing the wild type α_1-AT (*W*) and the variant Z (*Z*), Z/F51L (*ZT1*), Z/F51L/M374I (*ZT2*), Z/T59A/T68A/A70G (*ZT3*) were pulse-labeled with [^{35}S]methionine for 5 min and then chased with excess unlabeled methionine for the indicated time. The intracellular lysates were immunoprecipitated with the α_1-AT polyclonal antibody, and the obtained immunoprecipitates were analyzed by SDS-PAGE fluorography. *a*, unglycosylated form of α_1-AT; *b*, core-glycosylated form of α_1-AT. *B,* the yeast transformants expressing the wild type α_1-AT (*W*) and the variant S_{iiyama} (*Si*), S_{iiyama}/F51L (*SiT1*), S_{iiyama}/F51L/M374I (*SiT2*), and S_{iiyama}/T59A/T68A/A70G (*SiT3*) were pulse-chased and analyzed as described for *A*.

defect by the stable mutations.

The apparent ER retention of S_{iiyama} α_1-AT was not as obvious during the chase (Fig. 5*B*), implying rapid degradation. The ER form of the variant was detected with S_{iiyama}/T1 and S_{iiyama}/T2 during the chase, probably due to less degradation compared with the S_{iiyama} alone. Again the core-glycosylated S_{iiyama}/T3, like the Z/T3, was not detected. The nonglycosylated cytoplasmic form of S_{iiyama} was also degraded, though not so much as that of the Z type. The cytoplasmic form of S_{iiyama} carrying the thermostable mutations became more resistant to degradation (Fig. 5*B*).

DISCUSSION

In eukaryotes, secretory proteins that cannot fold correctly are generally retained within the ER, and the retained proteins are ultimately degraded or accumulate as insoluble aggregates (17–19). There are two fundamentally distinct reasons why the native conformation of a mutant protein is not produced: one is a kinetic block during folding and the other is a drastic loss of stability. Our present study suggests different causes for the defect of Z and S_{iiyama} variant α_1-AT.

Defective Folding Kinetics as a Cause of Secretion Block of the Z Variant—We have shown recently that the Z mutation causes a kinetic defect that leads to accumulation of a folding intermediate with a high tendency to aggregate (20). If the folding block of the Z variant α_1-AT is the primary cause of secretion block, it is to be expected that any mutations suppressing the kinetic retardation of the Z-type folding will relieve the secretion blockage. Various thermostable mutations suppressing the folding defect of the Z variant *in vitro* (Fig. 1) also suppressed the secretion defect (Fig. 3) as well as the intracellular degradation (Fig. 5) in the yeast secretion system. Significantly, our findings show that the extent of suppression in the secretion and degradation was proportional to the extent of suppression in the folding of Z protein. The results strongly support the notion that the increase in secretion efficiency of the Z variant α_1-AT is directly caused by the improved folding of the protein, which was conferred by the combined stable mutations.

The defective step during the folding of Z protein is likely to be the last stage of folding from a compact intermediate to the native form (20). How is the kinetic block of the Z type overcome by these stable equilibrium mutants? We have previously proposed a kinetic partitioning between the productive folding pathway and a kinetic trap (13). While the Z mutation induces partitioning into the folding trap, a more favorable partitioning into the productive pathway would be induced by the thermostable mutation. These mutations were shown to enhance the closure of A β-sheet, as revealed by the retardation of the mutant proteins in producing a complex with the peptide mimicking the sequence of reactive center loop (13), and by the delay in converting the native state into the latent state (11). The results suggest that the accumulated folding intermediate in the Z mutation is in a state in which A β-sheet is not completely closed and is likely to be a precursor of the loop-sheet polymers. Interestingly, the suppression of the folding and secretion defect of the Z type by T3 was more efficient than by T2, although T2 and T3 have the same conformational stability of $\Delta\Delta G$ (~3 kcal/mol) (11). It was noticed that urea dependence of the unfolding rate was much smaller for T3 than for T2 (11), indicating that the unfolding rate of T3 at physiological condition would be much greater that that of T2. Further biochemical characterization is required to provide a precise mechanism for the enhancement of the Z-type secretion by these stable mutation.

The conformational stability of the Z protein in urea, once folded successfully, appeared to be affected only slightly (20). A reduced association rate constant of the plasma Z α_1-AT with neutrophil elastase was attributed to the altered local conformation of the reactive center loop rather than the change in stability (21). The results in Table I also showed that most of the secreted Z proteins, but not the S_{iiyama} proteins, retain the inhibitory activity, indicating that the primary cause of the Z type retention in the ER is the folding retardation and not the stability defect.

Reduced Conformational Stability of S_{iiyama}—The native conformation of S_{iiyama} protein was not observed in the TUG gel system after 1 h *in vitro* translation (Fig. 1). Unlike the Z variant, however, the folded form of S_{iiyama} type was never produced even after a longer period of folding (data not shown), which suggests that the defect of S_{iiyama} might be the stability defect. Indeed, the stability loss of S_{iiyama} α_1-AT was so great that its defect could be suppressed only by T3 (Fig. 1). Even in this case the stability of the native S_{iiyama}/T3 form was much lower than that of the wild type, as revealed by the transition midpoint of the mutant protein shown in the TUG gels. This is quite a contrast to the result that the stability of the Z/T3

α_1-AT is as great as that of the T3 α_1-AT (Fig. 1, S_{iiyama}/T3 *versus* Z/T3). The Z mutation (E342K), being located at the protein surface, does not appear to induce a substantial loss of stability. In contrast, the conformational stability of S_{iiyama} (S53F), which is located at the hydrophobic core, appears to be drastically affected.

The secretion defect of S_{iiyama} could be rescued, though in a very low yield, by T1 and T2 mutations (Fig. 4), but the folding defect of S_{iiyama} could not be rescued by these mutations in the *in vitro* folding (Fig. 1). There is an obvious distinction between *in vivo* folding and *in vitro* folding. The *in vitro* folding of the nascent polypeptides is an equilibrium process between the native and the non-native species, but the *in vivo* folding is a vectorial process in that as soon as the secretion competent form is produced, it is pulled out of the folding-unfolding equilibrium into the secretory pathway. Thus, if the major cause for the defect of S_{iiyama} is the loss of stability and not the kinetic problem, the secretion of the mutant would be rescued as far as the stability loss is compensated to some degree, though it is not enough to show the native band on the TUG gel system. Such compensation may be enough for maintaining the secretion-competent state. The enhancement in the secretion of S_{iiyama} by T3 was not greater than that by T2, although the enhancement of the Z secretion by T3 was much greater than that by T2 (Table I). The results indicate that the folding defect of S_{iiyama} is intrinsically different from the Z-type folding defect. The cause of the folding defect of S_{iiyama} does not appear to be of kinetic origin because incubation of the S_{iiyama}/T3 at 30 °C prior to electrophoresis to allow further folding did not increase the production of the native form (data not shown), whereas the incubation of the Z type or the Z/T1 improved folding efficiency significantly (20). Further evidence for supporting the stability loss of S_{iiyama} came from the results that none of the successfully secreted S_{iiyama} α_1-AT carrying the stable mutations were active (Table I). It appears that the stability of these secreted α_1-AT was not great enough to confer inhibitory activity.

Our results on S_{iiyama} differ from those of Foreman and collaborators (9), in that the secretion defect of S_{iiyama} was suppressed by F51L in *Xenopus* oocyte more efficiently than that of the Z type. One possible explanation for this apparent inconsistency is that the defect of S_{iiyama} is more sensitive to certain factors like temperature and local protein concentration. We have observed that at a high temperature such as 37 °C the suppression on the secretion defect of S_{iiyama} by the thermostable mutation did not occur at all in the yeast system. This may also be related to the fact that the aggregation of S_{iiyama} α_1-AT is more severe than the Z type in the plasma (6). It is possible that since the culture temperature of *Xenopus* is much lower (~20 °C), the mutational effect of destabilization might have been reduced. On the other hand, the kinetic defect, if it existed, would have been magnified at a low temperature. Our results in the present study and the previous data from the *Xenopus* oocyte system (9) support strongly that the stability loss as opposed to the kinetic defect is the major cause of the folding and secretion defect of S_{iiyama}.

Intracellular Degradation of the Variant Polypeptides—The ER retention and subsequent degradation of the transport-impaired α_1-AT variants have been studied in various cell systems (22–25). The amounts of the intracellular level of the Z or S_{iiyama} protein measured by short pulse labeling of the recombinant yeast was not much different from that of the wild type (Figs. 3*A* and 4*A, IC*), whereas the steady-state level of the Z or S_{iiyama} protein in the total intracellular fraction was lower than that of the wild type (Figs. 3*B* and 4*A, IC*), indicating that the intracellular form of the Z or S_{iiyama} protein is degraded in the yeast cells. The preferential degradation of Z variant α_1-AT in the yeast exocytic pathway as shown in the present study has been also observed in another independent study (26). In the present yeast system, the unglycosylated cytoplasmic form of the variant proteins as well as the core-glycosylated form retained in the secretion pathway also underwent degradation (Fig. 5). This is strong evidence that the intracellularly retained form of these genetic variants is not the native state, which was also supported by *in vitro* folding studies (Fig. 1). Interestingly, the degradation pattern of S_{iiyama} protein was somewhat different from that of the Z type. The ER form of the Z type was retained and degraded slowly, while the ER form of S_{iiyama} degraded much more rapidly (Fig. 5). On the other hand, the cytoplasmic form of the Z type degraded much more rapidly than that of S_{iiyama} (Fig. 5). It is quite possible that the intracellularly accumulated forms of Z and S_{iiyama}, and consequently the pattern of their degradation, could be different because of distinct origins of their defects: the former produced from a trapped folding intermediate and the latter from a denatured native structure.

The ER serves as a protein folding compartment for secretory proteins and the role of the ER as a quality control system is now well established (17, 27). The involvement of chaperones in the ER, such as calnexin (28), was implicated in the retention and degradation of the secretion-incompetent α_1-AT variant in the ER (29, 30). However, the detailed molecular mechanism of intracellular degradation of Z or S_{iiyama} variant has yet to be elucidated. The degradation of intracellular Z and S_{iiyama} α_1-AT does not seem to involve vacuolar proteases, since the yeast strain employed in the present study has disruption of the *PEP4* gene, which results in quite depressed levels of the three major proteases, PrA (Pep4p), PrB, and PrC(CPY) (31).

In conclusion, the present study demonstrated that the folding and stability defect of the Z and S_{iiyama} variant α_1-AT is the fundamental cause of secretion block and intracellular degradation. The results present direct supporting evidence for the kinetic defect of folding associated with the Z variant and the stability loss associated with the S_{iiyama} variant. The defects can be corrected by other mutations that improve the folding efficiency and stability compensation of the variants, which appears to be needed for maintaining the secretion-competent state and protection against proteolysis. A protein folding defect that is accompanied by aggregation of the polypeptides has been tentatively identified as a cause of several other human diseases (32, 33). Our results demonstrate that although the apparent causes for the secretion block of the α_1-AT variants are similar, that is aggregation, the fundamental causes can be quite different.

REFERENCES

1. Huber, R., and Carrell, R. W. (1989) *Biochemistry* **28,** 8951–8966
2. Crystal, R. G. (1989) *Trends Genet.* **5,** 411–417
3. Gadek, J. E., Fells, G. A., Zimmerman, R. L., Rennard, S. I., and Crystal, R. G. (1981) *J. Clin. Invest.* **68,** 889–898
4. Stein, P. E., and Carrell, R. W. (1995) *Nat. Struct. Biol.* **2,** 96–113
5. Lomas, D. A., Evans, D. L, Finch, J. T., and Carrell, R. W. (1992) *Nature* **357,** 605–607
6. Lomas, D. A., Finch, J. T., Seyama, K., Nukiwa, T., and Carrell, R. W. (1993) *J. Biol. Chem.* **268,** 15333–15335
7. Lomas, D. A., Elliott, P. R., Sidhar, S. K., Foreman, R. C., Finch, J. T., Cox, D. W., Whisstock, J. C., and Carrell, R. W. (1995) *J. Biol. Chem.* **270,** 16864–16870
8. Graham, K. S., Le, A., and Sifers, R. N. (1990) *J. Biol. Chem.* **265,** 20463–20468
9. Sidhar, S. K., Lomas, D. A., Carrel, R. W., and Foreman, R. C. (1995) *J. Biol. Chem.* **270,** 8393–8396
10. Carrel, R. W., and Stein, P. E. (1996) *Biol. Chem. Hoppe-Seyler* **377,** 1–17
11. Lee, K. N., Park, S. D., and Yu, M.-H. (1996) *Nat. Struct. Biol.* **3,** 497–500
12. Kang, H. A., Nam, S. W., Kwon, K.-S., Chung, B. H., and Yu, M.-H. (1996) *J. Biotechnol.* **48,** 15–24
13. Kim, J., Lee, K. N., Yi, G.-S., and Yu, M.-H. (1995) *J. Biol. Chem.* **270,** 8597–8601
14. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 488–492
15. Kang, H. A., Schwelberger, H. G., and Hershey, J. W. B. (1993) *J. Biol. Chem.* **268,** 14750–14756
16. Matoba, S., Fukayama, J., Wing, R. A., and Ogrydziak, D. A. (1988) *Mol. Cell.*

Biol. **8,** 4904–4916
17. Hammond, C., and Helenius, A. (1994) *J. Cell Biol.* **126,** 41–52
18. Hurtley, S. M., and Helenius, A. (1989) *Annu. Rev. Cell Biol.* **5,** 277–307
19. Sifers, R. N. (1995) *Nat. Struct. Biol.* **2,** 355–357
20. Yu, M.-H., Lee, K. N., and Kim, J. (1995) *Nat. Struct. Biol.* **2,** 363–367
21. Lomas, D. A., Evans, D. L., Stone, S. R., Chang, W-S. W, and Carrell, R. W. (1993) *Biochemistry* **32,** 500–508
22. Le, A., Ferrel, G. A., Dishon, D. S., Le, Q.-Q. A., and Sifers, R. N. (1992) *J. Biol. Chem.* **267,** 1072–1080
23. Le, A., Graham, K. S., and Sifers, R. N. (1990) *J. Biol. Chem.* **265,** 14001–14007
24. Wu, Y., Whitman, I., Molmenti, E., Moore, K., Hippenmeyer, P., Perlmutter, D. H. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 9014–9018
25. Teckman, J. H., and Perlmutter, D. H. (1996) *J. Biol. Chem.* **271,** 13215–13220
26. McCraken, A. A., and Kruse, K. B. (1993) *Mol. Biol. Cell* **4,** 729–736
27. de Silva, A. M., Balch, W. E., and Helenius, A. (1990) *J. Cell Biol.* **111,** 857–866
28. Hammond, C., Braakman, I., and Helenius, A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 913–917
29. Le, A., Steiner, J. L., Ferrell, G. A., Shaker, J. C., and Sifers, R. N. (1994) *J. Biol. Chem.* **269,** 7514–7519
30. Ou, W.-J., Cameron, P. H., Thomas, D. Y., and Bergeron, J. J. M. (1993) *Nature* **364,** 771–776
31. Woolford, C. A., Noble, J. A., Garman, J. D., Tam, M. F., Innis, M. A., and Jones, E. W. (1993) *J. Biol. Chem.* **268,** 8990–8998
32. Thomas, P. J., Qu, B.-H,, and Pedersen, P. L. (1995) *Trends Biochem. Sci.* **20,** 456–459
33. Helms, L. R., and Wetzel, R. (1996) *J. Mol. Biol.* **257,** 77–86