Folding and Stability of the Z and S_{iiyama} Genetic Variants of Human \( \alpha_1 \)-Antitrypsin*

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Z (Glu\(^{342} \rightarrow \) Lys) and S_{iiyama} (Ser\(^{53} \rightarrow \) Phe) genetic variations of human \( \alpha_1 \)-antitrypsin (\( \alpha_1 \)-AT) cause a secretion blockage in the hepatocytes, leading to \( \alpha_1 \)-AT deficiency in the plasma. Using in \textit{vivo} 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 \( \alpha_1 \)-AT, we investigated in \textit{vivo} folding and stability of these variant \( \alpha_1 \)-AT using the secretion system of yeast \textit{Saccharomyces cerevisiae}. Various thermostable mutations suppressing the folding block of the Z variant in \textit{vivo} 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 \textit{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} \( \alpha_1 \)-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.

The structural basis of aggregation of the variant \( \alpha_1 \)-AT has been suggested to be the loop-sheet polymerization in which a partial opening of the central \( \beta \)-sheet, the A \( \beta \)-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 \( \alpha_1 \)-AT. This implication is based on the finding that the secretion blockage of the Z or S_{iiyama} was efficiently suppressed in the \textit{Xenopus oocyte} system either by a thermostable mutation (Phe\(^{51} \rightarrow \) Leu; F51L) that enhanced the closure of a \( \beta \)-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 \textit{in vivo} aggregation. However, the mechanism by which each individual mutant \( \alpha_1 \)-AT tends to polymerize and thus cause secretion blockage remains unclear. One possible mechanism for the polymerization of the mutant \( \alpha_1 \)-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 \( \alpha_1 \)-AT, using \textit{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 \( \alpha_1 \)-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 \( \alpha_1 \)-AT protein has been described previously (12). Briefly, the recombinant \textit{Saccharomyces cerevisiae} Y2805 (MATa pep::HIS3 prb1::D1.6R can1 his3-200 ura3-52) harbors the \( \alpha_1 \)-AT expression vector, pYnuu-AT, that contains the cDNA of human \( \alpha_1 \)-AT fused in frame with the uninlusive signal sequence downstream of the GAL10 promoter. The yeast expression vectors for variant \( \alpha_1 \)-AT proteins were constructed by exchanging the 1.1-kilobase pair BglII/SalI fragment containing the coding sequence of \( \alpha_1 \)-AT (starting at 17th codon of mature \( \alpha_1 \)-AT) in pYnuu-AT with the corresponding fragment derived from the pBglIIAT plasmids that were used in the \textit{in vitro} transcription/translation.

**In Vitro Folding Assay**—To express the mutant forms of \( \alpha_1 \)-AT protein \textit{in vitro}, the cDNA fragment coding for the wild type \( \alpha_1 \)-AT in the plasmid pBglIIAT (13) was replaced with the corresponding cDNA fragment encoding the thermostable mutants derived from the human \( \alpha_1 \)-AT protein by oligonucleotide-directed mutagenesis (14). In \textit{in vitro} transcription and translation, the A \( \beta \)-sheet residues 51–53 of the Z variant are replaced by homologous residues of the Z variant, and the mutant residues are introduced to the Z variant. This effect is likely to depend on the misfolded A \( \beta \)-sheet residues, which are shown to be responsible for the secretion defect of the Z variant.
translation of the cloned gene were carried out and the translation products were analyzed by transverse urea gradient (TUG) gel as previously described (13).

Biochemical Labeling and Immunoprecipitation—The recombinant yeast cells were grown in methionine-labeling medium containing 2% galactose, 0.67% 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 [35S]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 suprenatant. 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 α1-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 ENHANCE (DuPont). For endoglycosidase H (endo H) treatment, imunoaffinity (PAGE). Gels were fixed, amplified, and fluorographed with immunoprecipitates were analyzed by 10% SDS-polyacrylamide 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 α1-AT more than F51L (T1) (10), a greater fraction (70–90%) 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 α1-AT carrying S1iymana 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 S1iymana was suppressed only by T3. About 20% of the S1iymana polypeptides were rescued to the native state by T3. The stability of the native S1iymana/T3 was much lower than that of T3, and even lower than that of the wild type.

FIG. 1. Suppression of the folding defect of Z and S1iymana variants by the thermostable mutations. The α1-AT proteins carrying the indicated mutations were synthesized in the presence of [35S]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.
Fig. 2. Expression and secretion of human $\alpha_1$-AT proteins from S. cerevisiae. A, kinetics of secretion of the wild type $\alpha_1$-AT in yeast. The yeast transformants expressing human $\alpha_1$-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 $\alpha_1$-AT. The immunoprecipitates were fractionated on 10% SDS-PAGE, followed by fluorography. Molecular mass markers are indicated in kilodaltons on the left. B, N-linked glycosylated forms of human $\alpha_1$-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 fluorography. a, unglycosylated form of $\alpha_1$-AT; b, core-glycosylated form of $\alpha_1$-AT; and c, hyperglycosylated form of $\alpha_1$-AT.

Secretion of Human $\alpha_1$-AT in Yeast System—As a model system to assess the effects of various mutations on the intracellular folding and secretion of $\alpha_1$-AT protein, a yeast expression system (12) with high-level secretion of biologically active human $\alpha_1$-AT into extracellular culture medium was utilized. To examine the kinetics of secretion of $\alpha_1$-AT in the recombinant yeast, the cells expressing the wild type human $\alpha_1$-AT were analyzed by pulse-chase and immunoprecipitation experiments. The immunoprecipitation of the obtained culture supernatants (Fig. 2A, EC) showed that significant amounts of the core-glycosylated $\alpha_1$-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 $\alpha_1$-AT protein. The level of secreted $\alpha_1$-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. 2A, IC) showed that a major polypeptide of ~46 kDa remained stable throughout the chase period. The intracellularly retained $\alpha_1$-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. 2B). Most of the unglycosylated form of $\alpha_1$-AT was found in the cytosolic fraction of yeast and the size of the unglycosylated $\alpha_1$-AT in yeast was slightly larger than that of $\alpha_1$-AT expressed in Escherichia coli (data not shown). The results imply that some portions of the wild type $\alpha_1$-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 $\alpha_1$-AT protein in a heterologous host system. The results in Fig. 2, however, show that major portions of the human $\alpha_1$-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 $\alpha_1$-AT on the Secretion Defect of Z and S iyama—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 $\alpha_1$-AT, the yeast transformants expressing $\alpha_1$-AT were pulse-labeled for 10 min with $[^{35}S]$methionine and analyzed by immunoprecipitation. There was no detectable Z-type $\alpha_1$-AT protein in the culture supernatant after the 10-min pulse (Fig. 3A, EC, lane Z). In combination with the thermostable mutations, however, some Z proteins were secreted into the culture media (Fig. 3A, 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 $\alpha_1$-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...
the wild type protein. The T2 mutation improved the secretion of Z protein to an 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. 3B). There was no significant difference in the relative amounts of the secreted Z proteins, as measured either by pulse labeling (Fig. 2B, EC) or by Western blot (Fig. 3B, 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_iyama variant was also blocked in the yeast secretion system (Fig. 4A, EC), and the secretion blockade 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_iyama type secretion was much lower. In Western blot (Fig. 4B, EC), the steady-state level of the secreted S_iyama carrying the various mutations was 5–20%, while that of Z type was 10–75% (Fig. 3B, EC). The T2 and T3 were more efficient than T1 in suppressing the secretion defect of S_iyama, 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 α1-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 α1-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 efficiency. In the case of S_iyama protein, however, the secreted proteins did not show any detectable inhibition activity, implying that the S_iyama proteins, even if successfully secreted outside cells, were not in the active native conformation.

| α1-AT proteins | Secretion yield | Inhibition activity |
|----------------|----------------|---------------------|
| W              | 100            | 100                 |
| ZT1            | 10             | 2                   |
| ZT2            | 25             | 17                  |
| ZT3            | 75             | 68                  |
| ST1            | 7              | 0                   |
| ST2            | 22             | 0                   |
| ST3            | 19             | 0                   |

The secretion level of α1-AT was estimated by densitometry scanning of the fluorograms in Figs. 3A and 4A. The secretion yield was calculated by dividing the amount of α1-AT in the medium by the total amount synthesized over the labeling period (cells + medium). The biological activity of α1-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 α1-AT. Designations of the mutants are the same as in Figs. 3 and 4.
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 ΔΔG (~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 α1-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_iyama 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_iyama—The native conformation of S_iyama 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_iyama type was never produced even after a longer period of folding (data not shown), which suggests that the defect of S_iyama might be the stability defect. Indeed, the stability loss of S_iyama α1-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_iyama/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
α1-AT is as great as that of the T3 α1-AT (Fig. 1, Siiyama/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 Siiyama (SS3F), which is located at the hydrophobic core, appears to be drastically affected.

The secretion defect of Siiyama could be rescued, though in a very low yield, by T1 and T2 mutations (Fig. 4), but the folding defect of Siiyama 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 vitro folding is a vекторial 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 Siiyama 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 Siiyama 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 Siiyama is intrinsically different from the Z-type folding defect. The cause of the folding defect of Siiyama does not appear to be of kinetic origin because incubation of the Siiyama/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/T3 improved folding efficiency significantly (20). Further evidence for supporting the stability loss of Siiyama came from the results that none of the successfully secreted Siiyama α1-AT carrying the stable mutations were active (Table I). It appears that the stability of these secreted α1-AT was not great enough to confer inhibitory activity.

Our results on Siiyama differ from those of Foreman and collaborators (9), in that the secretion defect of Siiyama 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 Siiyama 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 Siiyama 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 Siiyama α1-AT is more severe than that of 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 Siiyama.

Intracellular Degradation of the Variant Polypeptides—The ER retention and subsequent degradation of the transport-impaired α1-AT variants have been studied in various cell systems (22–25). The amounts of the intracellular level of the Z or Siiyama protein measured by short pulse labeling of the recombinant yeast was not much different from that of the wild type (Figs. 3A and 4A, IC), whereas the steady-state level of the Z or Siiyama protein in the total intracellular fraction was lower than that of the wild type (Figs. 3B and 4A, IC), indicating that the intracellular form of the Z or Siiyama protein is degraded in the yeast cells. The preferential degradation of Z variant α1-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 Siiyama 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 Siiyama degraded much more rapidly (Fig. 5). On the other hand, the cytoplasmic form of the Z type degraded much more rapidly than that of Siiyama (Fig. 5). It is quite possible that the intracellularly accumulated forms of Z and Siiyama, 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 α1-AT variant in the ER (29, 30). However, the detailed molecular mechanism of intracellular degradation of Z or Siiyama variant has yet to be elucidated. The degradation of intracellular Z and Siiyama α1-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 Siiyama variant α1-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 Siiyama 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 α1-AT variants are similar, that is aggregation, the fundamental causes can be quite different.

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