J. Biochem. 126, 584-590 (1999)

# Construction of a Screening System for Selecting Lysozyme Mutants Unable to Form a Stable Structure from Random Mutants

Kaori Kunichika, Yoshio Hashimoto, and Taiji Imoto[1]

*Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582*

Received April 22, 1999; accepted June 6, 1999

**To collect folding information, we screened and analyzed the recombinant hen lysozyme mutants which were not secreted from yeast. As model mutants, Leu8Arg, Ala10Gly, and Met12Arg were prepared by site-directed mutagenesis and analyzed as to whether they were secreted from yeast or not. Consequently, Ala10Gly was found to be secreted from yeast, but Leu8Arg and Met12Arg were not. Next, these mutants were expressed in *Escherichia coli* and refolded *in vitro*. As a result, Ala10Gly folded as the wild-type did. Leu8Arg efficiently refolded in renaturation buffer containing glycerol. Met12Arg did not refold even in the presence of glycerol. These results show that the Ala10Gly mutation does not affect folding or stability, that Leu8Arg is too unstable to be secreted from yeast, and that Met12Arg may be very unstable or the mutation affects the folding pathway. We screened the mutants that were not secreted by yeast from a randomly mutated lysozyme library, and obtained Asp18His/Leu25Arg and Ala42Val/Ser50Ile/Leu56Gln. These two mutants were expressed in *E. coli* and then refolded in the presence of urea or glycerol. These mutants were refolded only in the presence of glycerol. Each single mutant of Asp18His/Leu25Arg and Ala42Val/Ser50Ile/Leu56Gln was independently prepared and folded *in vitro*. The results showed that Leu25Arg and Leu56Gln were the dominant mutations, respectively, which cause destabilization. These results show that the mutant lysozymes which were not secreted from yeast may be unstable or have a defect in the folding pathway. Thus, we established a screening system for selecting mutants which are unable to form a stable structure from random mutants.**

**Key words: lysozyme, protein folding, random mutagenesis, screening, stability.**

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

Anfinsen showed that the tertiary structure of a protein is defined by its primary structure (*1*). This means that the primary structure contains the information for not only the tertiary structure but also the folding. Natural proteins have evolved to such primary structures that can attain the tertiary structures as well as the folding processes. That is, natural proteins have folding information. Deciphering of this information systematically is a good way of solving the problem regarding protein folding. It is known that proteins which cannot form stable conformations are not secreted (*2*). Thus, we examined the mutants which were not secreted from yeast.

Hen lysozyme is a stable enzyme consisting of a single polypeptide chain of 129 amino acid residues with a molecular mass of about 14.3 kDa. This enzyme is a proper model protein for obtaining structural information because its character is well known. We have established expression systems of mutated lysozyme in yeast and *Escherichia coli* (*3, 4*). Some approaches such as alanine-scanning (*5*) and site-directed mutagenesis are employed to analyze protein structure and function. On the other hand, random mutagenesis has been used to enhance or alter various features of enzymes, such as thermal stability (*6-11*), stability as to alkali (*12*), and substrate specificity (*13*). Random mutagenesis combined with a proper screening system could be a useful alternative for desired improvements of a protein's character and for *de novo* protein design.

Generally, randomly mutated genes are transferred to an expression vector after mutagenesis. In particular for the selection of a mutant without a specific phenotype, it is very important to delete clones which are devoid of the desired gene (*14-16*). This was the case in this work because we try to select the yeast clones that do not secrete the protein. Thus, we used the expression vector, which has the streptomycin-inactivating gene, and streptomycin-dependent *E. coli* (*17*). Using this system, the plasmid gene was found to contain 100% of the lysozyme gene.

In this work, we established a screening system involving yeast for obtaining mutants which cannot be secreted from yeast. This is the first report of *in vitro* analysis of clones which are not secreted from yeast.

[1] To whom correspondance should be addressed. Tel: +81-92-642-6662, Fax: +81-92-642-6667, E-mail: imoto@imm.phar.kyushu-u.ac.jp

Abbreviations: $Sm^d$, streptomycin dependence; $Sm^r$, streptomycin resistance.

© 1999 by The Japanese Biochemical Society.

## MATERIALS AND METHODS

*Materials*—Restriction enzymes, T4 polynucleotide kinase, T4 DNA polymerase, and T4 ligase were purchased from Takara Shuzo (Kyoto). Asahipak ES-502C and

Sephadex G-75 were obtained from Asahi Chemical Industry, and Pharmacia, respectively. Native hen egg-white lysozyme was donated by the QP Company (Tokyo). Wild-type lysozyme was expressed in *E. coli* as inclusion bodies, purified and refolded. Wild-type lysozyme has an extra Met residue at its N-terminal. *Micrococcus luteus*, a substrate of lysozyme, was purchased from Sigma Chemical, (St. Louis). Isopropyl $\beta$-D-thiogalactoside was purchased from Wako Pure Chemical. Oxidized glutathione and 2-mercaptoethanol were purchased from Nacalai Tesque (Kyoto). Dye Primer Cycle Sequencing Kits FS for DNA sequencing were purchased from PE Applied Biosystems Japan (Tokyo). The other chemicals used were of analytical or biochemical grade.

*Strains, Plasmids, Phage, and Antibodies*—The following bacterial strains, plasmids, and phage were used; *E. coli* RR1 (*18*), JM110 (*19*), CS3 (*20*), and KP3998 (*4*), bacteriophage M13mp19 (*19*), *E. coli* expression vector pKP1500 (*4*), yeast expression vector pKLZ99 (*3*), pAM82 (*21*), pHA394 (*17*), and *S. cerevisiae* AH22 (*21*). Preparation of LKS103 mouse anti hen-lysozyme IgG monoclonal antibodies was carried out according to the method of Maeda *et al.* (*22*).

*Construction of Wild-Type Lysozyme*—Wild-type lysozyme was expressed in *E. coli* as inclusion bodies (*4*).

*Construction of Lysozyme Mutants, Leu8Arg, Ala10Gly, and Met12Arg, as Model Proteins by Site-Directed Mutagenesis*—Site-directed mutagenesis was carried out by the method of Kunkel *et al.* (*23*). The primers used for the mutation of Leu8Arg, Ala10Gly, and Met12Arg were as follows: 5′-GATGTGAGCGAGCAGCGG-3′, 5′-AGCTAGCAGGGGCTATGAA-3′, and 5′-AGCGGCTAGGAAGCGTCA-3′, respectively. These mutations were performed by use of pKLZ99 as a template. The *Sal*I fragment was transferred to the *Xho*I site of yeast expression vector pAM82 and then transformation of *S. cerevisiae* AH22 was performed according to spheroplast method of Hinnen *et al.* (*24*). Cloning to *E. coli* expression vector pKP1500 was carried out by PCR. Primers 5′-CCCGAATTCTATGAAAGTCTTTGGACGATGTGAG-3′ and 5′-GGCGGATCCTCACAGCCGGCAGCCT-3′ were used to introduce *Eco*RI and *Bam*HI sites at the 5′ and 3′-ends of the lysozyme gene, respectively. The PCR products were digested with *Eco*RI and *Bam*HI, and then inserted to pKP1500.

*Construction of a Randomly Mutated DNA Library*—A randomly mutated DNA library was constructed according to the method of Horwitz and Dimaio (*25*). The distribution of mutations as a function of the level of contamination was calculated by the method of Derbyshire *et al.* for the mutagenic oligonucleotide (*26*). The concentration of the nucleotide mixture was set at 1%, approximately 33% of the oligonucleotides in the mixture being predicted to contain single base substitutions. The synthesized oligonucleotides were focused on the region of 11 amino acid residues. However, the oligonucleotides were synthesized with a further dozen bases at both termini in anticipation of a decrease in the mutation frequency at both ends. The structure of the mutagenic primer, RNLZ-1, was 5′-CTAAGATATCTGCAAAAGTCTTTGGACGATGTGAGCTAGCAGCGGCTATGAAGCGTCACG-3′ for the region of Lys1-Ala11, that of RNLZ-2 was 5′-CTAGCAGCGGCTATGAAGCGTCACGGCTTGATAACTATCGGGGATACAGCCTGGGAAAC-3′ for the region of Met12-Gly22, that of RNLZ-4 was 5′-GTGTGCGGCAAAATTCGAGAGTAACTTCAACACCCAGGCTACAAACCGTAACACCGATGG-3′ for the region of Phe34-Asn44, and that of RNLZ-5 was 5′-CCAGGCTACAAACCGTAACACCGATGGGAGTACCGACTACGGAATCCTACAGATCAACAG-3′ for the region of Arg45-Ile55. A randomly mutated DNA library was cloned to the yeast expression vector, pHA394, by digestion with *Sal*I and *Bam*HI (*17*). Transformation of *S. cerevisiae* AH22 was performed according to the spheroplast method of Hinnen *et al.* (*24*).

*Construction of the Asp18His, Leu25Arg, Ala42Val, Ser50Ile, and Leu56Gln Single Mutants*—Site-directed mutagenesis was carried out by the method of Kunkel *et al.* (*23*). The primers used for the mutation of Asp18His, Leu25Arg, Ala42Val, Ser50Ile, and Leu56Gln were as follows; 5′-CGTCACGGACTTCACAACTATCGGGGA-3′, 5′-CGGGGATACAGCAGAGGAAACTGGGTG-3′, 5′-TTCAACACCCAGGTTACAAA CCGTAAC-3′, 5′-AACACCGATGGGATTACCGACTACGGA-3′, and 5′-CTACGGAATCCAACAGATCAACAG-3′, respectively. Expression in *E. coli* was carried out using expression vector pKP1500 (*4*).

*Lytic Assaying on Plates for Random Mutants of Lysozyme*—Lytic assaying for screening was performed on an agar plate of the low phosphate Burkholder medium (0.6 mM $KH_2PO_4$) (*17*). The *PHO5* promoter on plasmid pHA394 is induced under low-phosphate growth conditions. The plate was overlaid with the substrate *M. luteus*, which was irradiated with ultraviolet rays. On the other hand, yeast transformants containing the lysozyme gene randomly mutagenized described above were prepared using the cloning system with the *rpsL*($Sm^d$)-*E. coli* and *addA*($Sm^r$)-genes. The yeast transformants were cultivated on the *M. luteus*-plate. The lytic haloes of clones were compared with that of the wild-type, the clones which did not form haloes being selected.

*Determination of the Secreted Protein Amounts by ELISA*—Determination of the amounts of secreted proteins was performed with an ELISA system according to the method of Buchner and Rudolph (*27*) with slight modifications. In each of the 96 wells of titration plates was placed 50 $\mu$l of a 5 $\mu$g/ml solution of rabbit anti hen-lysozyme polyclonal antibodies in 0.05 M sodium carbonate buffer (pH 9.6) containing 3.1 mM $NaN_3$. The plates were kept at 4°C overnight. After removal of supernatants, 100 $\mu$l of 10% skim milk was added, followed by standing at room temperature for 1 h. The plates were washed three times with Tris-Tween buffer (10 mM Tris-HCl buffer containing 0.05% Tween 20, 3.1 mM $NaN_3$ and 136.9 mM NaCl, at pH 7.2-7.4), and then 50 $\mu$l of the yeast culture supernatant was placed in each well. After standing at room temperature for 1 h, the plates were washed three times with Tris-Tween buffer. After the addition of 50 $\mu$l of a 1 $\mu$g/ml solution of LKS103 mouse anti hen-lysozyme IgG monoclonal antibodies, the plates were allowed to stand at room temperature for 1 h. After three washes with Tris-Tween buffer, 50 $\mu$l of alkaline phosphatase-goat anti-mouse IgG+A+M (H+L) was added. After standing at room temperature for 0.5 h, the plates were washed four times with Tris-Tween buffer. To the plates was added 100 $\mu$l of a staining solution containing disodium *p*-nitrophenylphosphate hexahydrate. The staining reaction was carried out at room temperature for 1 h. The absorbance at 405 nm



Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

was measured with a Bio Rad model 450 microplate reader.

*Analyses of Plasmids from Yeast Transformants Which Did Not Secrete Lysozyme*—As for yeast transformants which did not secrete lysozyme, plasmid DNA was separated from yeast cells (*28, 29*), followed by both *Sal*I and *Bam*HI digestion, and analysis by agarose gel electrophoresis.

*Expression of Lysozyme in E. coli*—The lysozyme gene, which was selected by means of the yeast secretory system, was cloned in *E. coli* expression vector pKP1500 and then expressed in *E. coli* according to the method of Imoto *et al.* (*30*).

*Purification of Lysozyme Expressed in E. coli*—Purification of the wild-type and mutant lysozymes produced in *E. coli* was carried out according to the method of Mine *et al.* (*31*). Harvested cells were suspended in 3-(*N*-morpholino) propanesulfonic acid buffer, pH 7, and then sonicated exhaustively. After the mixture had been centrifuged, the precipitate was digested with DNase. After the mixture had been centrifuged, the precipitate was suspended in a 6 M Gdn-HCl solution (0.575 M Tris-HCl buffer containing 6 M Gdn-HCl and 5.25 mM EDTA, pH 8.6) for reduction. After the solution had been degassed, it was reduced with 2-mercaptoethanol under a nitrogen atmosphere at 40°C for 1 h. The reduction mixture was diluted with 10% acetic acid to extract the reduced lysozyme. The supernatants were chromatographed on a 1.5 cm × 150 cm column of Sephadex G-75, which was eluted with 10% acetic acid. The main peak fractions were collected and lyophilized to give fairly pure lysozyme as an inactive form. The purity was judged to be ≥90% on SDS-PAGE (data not shown).

*Renaturation of Reduced Lysozyme by Means of Rapid Dilution*—Renaturation of lysozyme was carried out according to the method of Ueda *et al.* (*32*). Purified lysozyme derivatives were dissolved in 4.5 ml of a 8 M urea solution for reduction. After the solution had been degassed, 25 $\mu$l of 2-mercaptoethanol was added to the solution, and then the solution was incubated at 40°C for 1 h under a nitrogen atmosphere (reduced solution). To the reduced solution, 40.5 mg of oxidized glutathione dissolved in 500 $\mu$l of the 8 M urea solution was added (redox solution). Separately, 9.9 ml of the renaturation buffer was preincubated at 40°C. Renaturation of lysozyme derivatives was initiated by adding 250 $\mu$l of the redox solution to the renaturation buffer containing respective concentration of urea or glycerol with stirring at room temperature.

*Measurement of the Folding Yield by HPLC Analysis*—For cation-exchange HPLC analysis, an aliquot of respective renatured solution was applied directly to the column (Asahipak ES-502C, 7.6 × 100 mm). The column was eluted with a gradient formed from 30 ml of 0.05 M phosphate buffer (pH 7.0) and 30 ml of 0.05 M phosphate buffer (pH 7.0) containing 0.5 M NaCl at the flow rate of 1.0 ml/min. The amount of the folded lysozyme derivative was determined by comparing the peak area with that of a known concentration of native lysozyme.

*Measurement of the Folding Yield as Lytic Activity*—The activities of the folded lysozyme derivatives against *M. luteus* were determined turbidimetrically at 450 nm in 0.05 M potassium phosphate buffer, pH 7.0 at 30°C (*33*). The amount of the folded lysozyme derivative was determined by comparing the initial rate of decrease with that of a known concentration of native lysozyme.

TABLE I. **Possible lysozyme mutants produced by one-point mutation for residues 1-14.**

| Residue number | Original amino acid | Codon | Possible mutaiton[a] |
|---|---|---|---|
| 1 | K | AAA | E,I,N,Q,R,T,*[b] |
| 2 | V | GTC | A,D,F,G,I,L |
| 3 | F | TTT | C,I,L,S,V,Y |
| 4 | G | GGA | A,E,R,V,* |
| 5 | R | CGA | G,L,P,Q,* |
| 6 | C | TGT | F,G,R,S,W,Y,* |
| 7 | E | GAG | A,D,G,K,V,Q,* |
| 8 | L | CTA | I,P,Q,R,V |
| 9 | A | GCA | E,G,P,S,T,V |
| 10 | A | GCG | E,G,P,S,T,V |
| 11 | A | GCT | D,G,P,S,T,V |
| 12 | M | ATG | I,K,L,R,T,V |
| 13 | K | AAG | E,M,N,Q,R,T,* |
| 14 | R | CGT | C,G,H,L,P,S |

[a]Possible mutation produced by one-point mutation. [b]* (asterisk) indicates stop codon.

## RESULTS

*Design of Leu8Arg, Ala10Gly, and Met12Arg as Model Mutants for Construction of a Screening System*—When random mutagenesis was carried out for region Lys1-Arg14, mutants which would hinder folding were estimated. Mutants were selected from possible single mutants for the residue 1-14 region (Table I). Leu8Arg, Ala10Gly, and Met12Arg were prepared. The locations of the mutation sites are shown in Fig. 1. Leu8 is located in the $\alpha$-helix and buried in the interior. Due to the change from Leu8 to Arg, which has a large side-chain and a positive charge, the Leu8Arg mutant lysozyme would not retain the folded structure. Ala10 is located in the center of the $\alpha$-helix. Due to the change from Ala10 to Gly, which is a helix breaker, the Ala10Gly mutation might affect the helix structure and unfold the protein. Met12Arg was prepared for a similar reason to Leu8Arg.

*Secretion from Yeast and Folding of Mutants, Leu8Arg, Ala10Gly, and Met12Arg*—Leu8Arg, Ala10Gly, and Met12Arg were expressed by the yeast system and examined as to whether they form lysis haloes. Ala10Gly

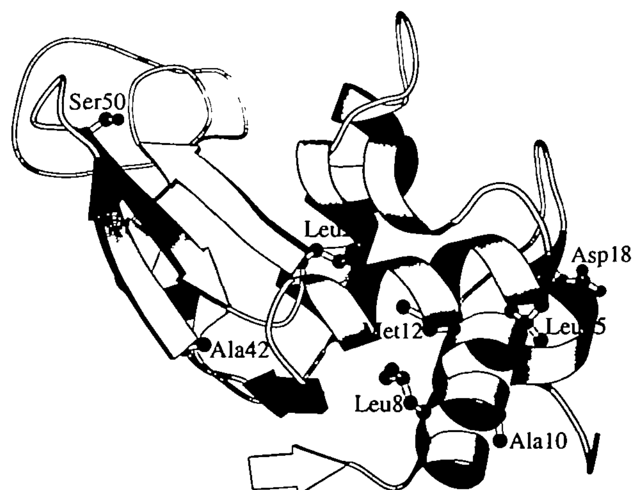


Fig. 1. **Ribbon model for the structure of lysozyme.** This figure was constructed with the MOLSCRIPT program.



Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

formed a lysis halo, but Leu8Arg and Met12Arg did not. Next, the amounts of secreted mutants were measured by ELISA. Ala10Gly was secreted normally, and Leu8Arg and Met12Arg were not secreted at all. The wild-type, Leu8-Arg, Ala10Gly, and Met12Arg were expressed in *E. coli*. After purification of these proteins from inclusion bodies, the unfolded proteins were renatured in the presence of 2 M urea, 1 M urea, water, 10% glycerol, and 30% glycerol by rapid dilution. The folding yield of each mutant and the wild-type was estimated by lytic activity analysis and cation exchange HPLC analysis (Tables V and VI). The wild-type was refolded in an about 40% yield under the optimum conditions by rapid dilution (*22*). The folding yield of Ala10Gly remained as high as that of the wild-type in the presence of either urea or glycerol. These results were correlated with those of yeast-screening *in vivo*. The folding yield of Leu8Arg was very low except in the presence of glycerol. Met12Arg was not folded in the presence of either urea or glycerol.

*Design of the Oligonucleotide for the Construction of a Random Mutant Library*—The primary sequence of lysozyme (129 amino acid residues) was divided into 11 residue regions. Random mutagenesis as to each of regions Lys1-Ala11, Met12-Gly22, Phe34-Asn44, and Arg45-Ile55 was carried out in this study. The primary sequence of these regions has an $\alpha$-helix, a $\beta$-sheet and catalytic residues, Glu35 and Asp52. Then, a random library which maximizes one point mutation was prepared for each region ("MATERIALS AND METHODS"). Possible amino acid substitutions produced by one point mutations are shown in Table I.

*Introduction of Random Mutagenesis*—The introduction of random mutagenesis was performed by the method of Horwitz and Dimaio (*25*). The numbers of independent clones obtained for each region are shown in Table II. With the method of Hutchinson III *et al.* (*34*), the number of clones to be screened to cover 90% of all mutations in one region is about 1,550. However, 1,550 clones were not covered here (Table III, step 1), because the purpose of this work was to establish a screening system.

*Introduction to Yeast-E. coli Shuttle Vector pHA394*—pHA394, derived from yeast expression vector pAM82 (*21*), contains an *aadA*($Sm^r$)-gene (3.6 kb) between the *Sal*I and *Bam*HI sites. By using the streptomycin-dependent *E. coli* and streptomycin-resistant gene system, only clones in which the streptomycin-resistant gene is replaced by the desired genes can be selected. The random DNA library prepared was inserted between the *Sal*I and *Bam*HI sites of pHA394. *rpsL*($Sm^d$)-*E. coli* CS3 was transformed with the pHA394 plasmids containing random lysozyme genes in the presence of both streptomycin and ampicillin, and yeast was transformed with the plasmids from the CS3 transformants.

Screening of yeast transformants was performed in two steps. In the first step, the clones which do not form lytic haloes were selected (Table III). In the next step, the clones obtained in the first step were further selected by ELISA, because the clones seleted by means of the lysis assay may contain lysozymes without lytic activity (Table III).

*Identification by Means of the DNA and Amino Acid Sequences*—RR1 was transformed with the plasmid DNA from the screened yeast clone (Table III, step 4). For each region, the number of clones in Table III, step 4, decreased as compared with the clones in Table III, step 3. This is because only a part of the clones in Table III, step 3, were transformed and analyzed. Plasmid DNA from the screened yeast clone was digested with *Sal*I and *Bam*HI. Clones haboring lysozyme fragments (about 450 bp) were selected (Table III). The number of clones in Table III, step 5, was considerably decreased as compared with the number in Table III, step 4. The reasons are considered to be as follows: (i) contamination of chromosomal DNA during DNA cloning, and (ii) accidental occurrence of a *Sal*I site or

TABLE II. **Number of independet clones for each region.**

| | Mutagenesis total clones[a] | *E. coli* CS3 transformation total clones[b] |
|---|---|---|
| Lys1-Ala11 | 1,800 | 6,780 |
| Met12-Gly22 | 2,025 | 9,570 |
| Phe34-Asn44 | 4,160 | 19,000 |
| Arg45-Ile55 | 13,000 | 19,000 |

[a]All independent clones in the M13 phage library. [b]All independent clones of CS3 transformants containig pHA394 derivatives.

TABLE III. **Results of 1st and 2nd screening for the random library.**

| | Lys1-Ala11 | Met12-Gly22 | Phe34-Asn44 | Arg45-Ile55 |
|---|---|---|---|---|
| (step 1) Number of total colonies screended | 1,120 | 1,000 | 400 | 500 |
| (step 2) Number of colonies in the 1st screening[a] | 73 | 73 | 70 | 75 |
| (step 3) Number of colonies in the 2nd screening[b] | 27 | 47 | 47 | 58 |
| (step 4) Number of colonies RR1 transformed | 22 | 43 | 19 | 10 |
| (step 5) Number of colonies showing lysozyme fragment on *Sal*I and *Bam*HI digestion | 4 | 8 | 7 | 8 |

[a]1st screening···Lytic haloes on the plate ("MATERIALS AND METHODS"). [b]2nd screening···Determination of the secreted protein amounts by ELISA ("MATERIALS AND METODS").

TABLE IV. **Results of DNA sequencing.**

| Region | Sample name | Base change | Amono acid substitution |
|---|---|---|---|
| Lys1-Ala11 | KU5-1 | 11(GCT) to GC⊿ | |
| | | 3(TTT) to TGT | Phe3 to Cys |
| | KU5-172 | 9(GCT) to ⊿CA | |
| Met12-Gly22 | KU6-292 | 13(AAG) to TAG | stop codon |
| | | 22(GGA) to AGA | Gly22 to Arg |
| | KU6-670 | 18(GAT) to CAT | Asp18 to His |
| | | 25(CTG) to CGG | Leu25 to Arg |
| Phe34-Asn44 | KU3-43 | 16(GGA) to AGA | Gly16 to Arg |
| | | 72(TCC) to TAC | Ser72 to Tyr |
| | KU3-101 | 29(GTG) to GTC | Val29 to Val |
| | | 34(TTC) to TTA | Phe34 to Leu |
| | | 50(AGT) to TGT | Ser50 to Cys |
| | | 51(ACC) to GCC | Thr51 to Ala |
| Arg45-Ile55 | KU4-14 | 42(GCT) to GTT | Ala42 to Val |
| | | 50(AGT) to ATT | Ser50 to Ile |
| | | 56(CTA) to CAA | Leu56 to Gln |
| | KU4-17 | 53(TAC) to TAA | stop codon |



Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

TABLE V. **Folding yields of mutant lysozymes detected as lytic activity[a] under the conditions of various concentrations of urea or glycerol.**

| Mutant | 2 M urea | 1 M urea | 0 M urea | 10% glycerol | 30% glycerol |
|---|---|---|---|---|---|
| Native[b] | 84.0 | 81.3 | 80.4 | 65.2 | 72.4 |
| Wild[c] | 41.7 | 43.6 | 38.5 | 34.7 | 36.6 |
| Leu8Arg | 1.1 | 2.2 | 2.2 | 20.5 | 34.8 |
| Ala10Gly | 31.3 | 32.1 | 33.0 | 31.3 | 33.9 |
| Met12Arg | 0.8 | 1.0 | 0.9 | 0.6 | 1.1 |
| Ala42Val/Ser50Ile/Leu56Gln | 0.4 | 1.1 | 2.0 | 8.6 | 25.0 |
| Asp18His/Leu25Arg | 0.2 | 0.2 | 0.5 | 0.5 | 1.3 |
| Asp18His | 74.6 | 74.6 | 53.7 | 73.7 | 77.3 |
| Leu25Arg | 1.7 | 2.5 | 4.2 | 19.5 | 28.0 |
| Ala42Val | —[d] | — | — | — | — |
| Ser50Ile | — | — | — | — | — |
| Leu56Gln | — | — | — | — | — |

[a]Lytic activity of the folded lysozyme derivatives against *M. luteus* was determined turbidimetrically at 450 nm in 0.05 M potassium phosphate buffer pH 7.0 at 30°C (*33*). [b]This means native hen lysozyme purified from hen egg. [c]This means the recombinant wild-type lysozyme produced in *E. coli*. [d]Not determined with this method.

TABLE VI. **Folding yields of mutant lysozymes detected by ion-exchange HPLC under the conditions of various concentrations of urea or glycerol.**

| Mutant | 2 M urea | 1 M urea | 0 M urea | 10% glycerol | 30% glycerol |
|---|---|---|---|---|---|
| Native[a] | 73.9 | 83.8 | 75.2 | 62.1 | 74.9 |
| Wild[b] | 35.5 | 48.7 | 27.9 | 23.2 | 26.9 |
| Leu8Arg | 0 | 2.9 | 7.2 | 24.0 | 39.2 |
| Ala10Gly | 42.1 | 54.3 | 38.8 | 45.2 | 47.3 |
| Met12Arg | 0 | 0 | 0 | 0 | 0 |
| Ala42Val/Ser50Ile/Leu56Gln | 0 | 0 | 2.2 | 8.9 | 17.8 |
| Asp18His/Leu25Arg | 0 | 1.4 | 5.4 | 8.2 | 14.9 |
| Asp18His | 31.8 | 35.4 | 22.6 | 24.6 | 24.6 |
| Leu25Arg | 0 | 0 | 5.0 | 12.0 | 12.5 |
| Ala42Val | 28.5 | 34.7 | 23.1 | 21.3 | 19.5 |
| Ser50Ile | 8.9 | —[c] | 8.2 | — | 10.6 |
| Leu56Gln | 0 | — | 2.2 | — | 26.6 |

[a]This means native hen lysozyme purified from hen egg. [b]This means the recombinant wild-type lysozyme produced in *E. coli*. [c]Not determined with this method.

*Bam*HI site in the lysozyme gene. As a result of these two reasons, various sizes of DNA fragments were obtained. The mutations were identified by DNA sequencing (Table IV). In two mutants, deletion of bases occurred. On KU2-1, Phe3 was substituted with Cys and the thymidine in the GCT codon corresponding to residue 11 was deleted. On KU5-172, the guanine in the GCA codon corresponding to residue 9 was deleted. Consequently, it was considered that lysozyme could not be synthesized by the frameshift mutation. The mutations in KU3-43 and KU3-101 were out of the desired region. These were considered to be unexpected mutations occurring during cloning. On KU6-292, the AAG codon corresponding to residue 13 was changed to a stop codon (amber mutation). On KU4-17, the TAA codon corresponding to residue 53 was changed to stop codon (ochre mutation). The only clones with reasonable mutations were KU6-670 (Asp18His/Leu25Arg) and KU4-14 (Ala42Val/Ser501Ile/Leu56Gln).

*Expression of Mutant Lysozyme in E. coli and Its Folding by Means of Rapid Dilution*—In order to analyze the mutants selected with the yeast screening system, the proteins were expressed in *E. coli*, purified as denatured proteins, and renatured into active structures as performed in model mutants.

Two mutants selected from the random library were renaturated under the conditions of 2 M urea, 1 M urea, water, 10% glycerol, and 30% glycerol. The folding yield of each mutant and wild-type was estimated by lytic activity and cation exchange HPLC analyses (Tables V and VI). Ala42Val/Ser501Ile/Leu56Gln was not refolded in the presence or absence of urea. However, the mutant was refolded in the presence of glycerol. These results show that Ala42Val/Ser501Ile/Leu56Gln is unstable in water. As to Asp18His/Leu25Arg, the folding yield expressed as lytic activity was very low under all conditions of urea, water, and glycerol. As judged on cation exchange HPLC, Asp18His/Leu25Arg did not refold in the presence of 2 M urea at all, and the peak was very low in the presence of 1 M urea and in water. However, a clear peak was detected in the presence of glyceol. The folding yield of Asp18His/Leu25Arg was very low (<5%) in the presence and absence of urea. However, the folding yield of the unfolded protein increased as the concentration of glycerol did. The folding yield expressed as lytic activity was correlated to that on cation exchange HPLC. These results show that both Ala42Val/Ser501Ile/Leu56Gln and Asp18His/Leu25Arg are unstable mutants in water.

*Folding of Each Independent Mutant, Asp18His/Leu25Arg and Ala42Val/Ser501Ile/Leu56Gln, by Means of Rapid Dilution*—Each independent single mutant of Ala42Val/Ser501Ile/Leu56Gln and Asp18His/Leu25Arg was prepared and folded *in vitro* under the conditions of urea, water and glycerol (Tables V and VI). Asp18His was refolded with a yield of 20-35% under all conditions. The high lytic activity observed for this mutant might reflect the increased basidity of it. Leu25Arg was not refolded in the presence of urea or in water. However, in the presence of glycerol, the folding yield of Leu25Arg was increased. These results show that Leu25Arg is unstable in water. Ser42Val was refolded under all conditions of urea, water and glycerol. The folding yield of Ser50Ile was low but about 10% under all conditions. Leu56Gln was not refolded in the presence of urea and very low in water. However, in the presence of glycerol, the folding yield was 27%.

## DISCUSSION

In this work, we established a screening system for selecting mutant lysozymes which have a defect in folding or stability.

Secretory proteins are synthesized on the endoplasmic reticulum (ER) membrane, and pass through the ER membrane. Structural formation, in other words protein folding, occurs in the lumen of the ER (*35, 36*). Since proteins are promptly transported from the ER to the Golgi upon folding, misfolded secretory proteins are retained in the ER by a conformational proofreading apparatus and eventually decomposed (*37*). Although it is clear that grossly misfolded proteins are retained by the proofreading pathway, the limits of detection with this system are not yet known. By the proofreading system, secretory proteins can not be secreted into an extracellular compartment without maintenance of their tertiary structure in the ER. In our work, the secretory signal sequence was introduced



Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

before the hen lysozyme gene, and wild-type hen lysozyme was definitely secreted from yeast (*3*). Thus, information on the critical residues that are essential for the folding can be obtained by selecting clones which are not secreted from yeast with a yeast expression system of lysozyme.

Randomly mutated lysozyme genes were inserted into an expression vector for screening by using the cloning system that Hashimoto *et al.* had already constructed (*17*). This system is based on the principle that *rpsL*(Sm$^d$)-*E. coli* carrying the *addA*(Sm$^r$) gene can not even grow in the presence of streptomycin. Only after the *addA* gene on the expression vector was replaced with the lysozyme gene, could *rpsL*(Sm$^d$)-*E. coli* grow. Thus, the transformants would definetely contain the lysozyme gene. For the selection of a mutant without a specific phenotype, it is very important to delete clones which are devoid of the desired gene. In this sense, this system is very useful.

With the method of Horwitz *et al.* (*25*), the number of clones to be screened to cover 90% of all mutations in one region is about 1,550. In this work, 1,120, 1,000, 400, and 500 yeast clones were screened in the regions of Lys1-Ala11, Met12-Gly22, Phe34-Asn44, and Arg45-Ile55, respectively. As a result of the screening, Ala42Val/Ser50-1Ile/Leu56Gln and Asp18His/Leu25Arg were selected, and then analyzed *in vitro*. The number of clones screened was less than 1,550 in this work. More useful mutants will be obtained by increasing the number of clones screened in each region.

The aggregation during refolding of proteins is strongly depressed in the presence of an appropriate concentration of urea (*38-40*), resulting in an increase in the folding yield of the unfolded protein. Since urea has the ability to dissolve hydrophobic side chains and main chain atoms, the solubility of the denatured protein increases (*41*). Therefore, non-specific interaction of the denatured protein is expected to decrease in the presence of urea. On the other hand, the presence of glycerol increases protein stability (*42-44*). The equilibrium between the native and denatured states would shift to the native state on the addition of glycerol. In the previous studies, the three-disulfide derivatives of lysozyme were effectively refolded in the presence of glycerol (*45, 46*). This knowledge showd that the gap in the energy level between the native and denatured states of Leu8Arg was smaller than that in the case of the wild-type, and that the addition of glycerol increased the gap in the energy level between the native and denatured states. Thus, Leu8Arg was recognized to be an unstable mutant. Met12Arg was not refolded in the presence of urea and glycerol. This meant that this mutant was not even stable in the presence of 30% glycerol, or that the change from Met to Arg was critical for the folding pathway.

Two mutants, Ala42Val/Ser501Ile/Leu56Gln and Asp18His/Leu25Arg, which were not secreted from yeast, were selected from a random library (Fig. 1). In the *in vitro* experiment, these two mutants are recognized to be unstable mutants. Each independent mutant of Ala42Val/Ser50Ile/Leu56Gln was prepared and folded *in vitro*. Ala42Val was refolded under all the conditions. The folding yield of Ser50Ile was low under all the conditions (about 10%). This suggests that Ser50Ile may affect both folding and stability. Ser50 is located in the $\beta$-sheet and is completely buried. Ser50 is the completely conserved residue in various lysozymes. Leu56Gln was only refolded in the presence of glycerol. This show that Leu56Gln causes destabilization. The substitution of Leu56 to Gln might cause destabilization by decreasing the hydrophobicity. Thus, the mutations in Ala42Val/Ser50Ile/Leu56Gln would cause destabilization.

Each independent single mutant of Asp18His/Leu25Arg was prepared and folded *in vitro*. The lytic activity of Asp18His showed it was reasonably well refolded. Leu25-Arg was refolded only in the presence of glycerol. These results show that Leu25Arg is the dominant mutation that causes destabilization. The helix has a dipole moment with the positive pole at the N-terminus of the helix (*47*). The electrostatic interaction between a charge and a helix dipole should take part in the stabilization of a protein (*48*). Leu25 is located near the N-terminus of the $\alpha$-helix as Asp18 is (Fig. 1). Conversion of Leu25 to Arg represents the change from a hydrophobic to a positive character. The side-chain volume of Arg is larger than that of Leu. These characters of Arg seem to affect the formation of the $\alpha$-helix. The Asp18His mutant showed high lytic activity, but the Asp18His/Leu25Arg mutant hardly retained any lytic activity. However, on cation exchange HPLC analysis, Asp18His/Leu25Arg was found to be clearly refolded in the presence of glycerol. These results indicated that the mutation of Asp18His/Leu25Arg might cause a structural change around the active site.

By using a yeast and *E. coli* expression system, we found that mutants which was not secreted from yeast had defects in structural stability or folding. There are two reasons why the mutants were not secreted from yeast. (i) The mutants are very unstable, and (ii) they have defects in the folding pathway. There has been no report that proteins which are not secreted from yeast have been analyzed *in vitro* using an *E. coli* expression system. In this work, a screening system was constructed for selecting mutants which are unable to form a stable structure from a random library of lysozyme. It was confirmed that useful information concerning protein structure could be obtained by analyzing clones which were not secreted from yeast.

## REFERENCES


1. Anfinsen, C.B. (1973) Principles that govern the folding of protein chains. *Science* **181**, 223-230
2. Kowalski, J.M., Parekh, R.N., and Wittrup, K.D. (1998) Secretion efficiency in *Saccharomyces cerevisiae* of bovine pancreatic trypsin inhibitor mutants lacking disulfide bonds is correlated with thermodynamic stability. *Biochemistry* **37**, 1264-1273
3. Hashimoto, Y., Yamada, K., Motoshima, H., Omura, T., Yamada, H., Yasukochi, T., Miki, T., Ueda, T., and Imoto, T. (1996) A mutation study of catalytic residue Asp 52 in hen egg lysozyme. *J. Biochem.* **119**, 145-150
4. Miki, T., Yasukochi, T., Nagatani, H., Furuno, M., Orita, T., Yamada, H., Imoto, T., and Horiuchi, T. (1987) Construction of a plasmid vector for the regulatable high level expression of eukaryotic genes in *Escherichia coli*: an application to overproduction of chicken lysozyme. *Protein Eng.* **1**, 327-332
5. Yu, M.H., Weissman, J.S., and Kim, P.S. (1995) Contribution of individual side-chains to the stability of BPTI examined by alanine-scanning mutagenesis. *J. Mol. Biol.* **249**, 388-397
6. Hecht, M.H., Sturtevant, J.M., and Sauer, R.T. (1984) Effect of stable amino acid replacements on the thermal stability of $NH_2$-terminal domain of phage l repressor. *Proc. Natl. Acad. Sci. USA* **81**, 5685-5689
7. Alber, T. and Wozniak, J.A. (1985) A genetic screen for mutations that increase the thermal stability of phage T4 lyso-




Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012

zyme. *Proc. Natl. Acad. Sci. USA* **82**, 747-750
8. Matsumura, M. and Aiba, S. (1985) Screening for thermostable mutant of kanamycin nucleotidyl transferase by the use of a transformation system for a thermophile, *Bacillus stearothermophilus*. *J. Biol. Chem.* **260**, 15298-15303
9. Lio, H., Mckenzie, T., and Hageman, R. (1986) Isolation of a thermostable enzyme varitant by cloning and selection in a thermophile. *Proc. Natl. Acad. Sci. USA* **83**, 576-580
10. Makino, Y., Negoro, S., Urabe, I., and Okada, H. (1989) Stability-increasing mutants of glucose dehydrogenase from *Bacillus megaterium* IWG3. *J. Biol. Chem.* **264**, 6381-6385
11. Joyet, P., Declerck, N., and Gaillardin C. (1992) Hyperthermostable variants of a highly thermostable alpha-amylase. *Bio/Technology* **10**, 1579-1583
12. Cunningham, B.C. and Wells, J.A. (1987) Improvement in the alkaline stability of subtilisin using an efficient random-mutagenesis and screening procedure. *Protein Eng.* **1**, 319-325
13. Olphant, A.R. and Struhl, K. (1989) An efficient method for generating proteins with altered enzymatic properties: Application to $\beta$-lactamase. *Proc. Natl. Acad. Sci. USA* **86**, 696-700
14. Hashimoto-Gotoh, T., Tsujimura, A., Kuriyama, K., and Matsuda, S. (1993) Construction and characterization of new host-vector systems for the enforcement-cloning method. *Gene* **137**, 211-216
15. Bernard, P., Kezdy, K.E., Melderen, L.V., Steyaert, J., Wyns, L., Pato, M.L., Higgins, P.N., and Couturier, M. (1993) The F plasmid CcdB protein induces efficient ATP-dependent DNA cleavage by gyrase. *J. Mol. Biol.* **234**, 534-541
16. Yazynin, S.A., Deyev, S.M., Jucovic, M., and Hartly, R.W. (1996) A plasmid vector with positive selection and directional cloning based on a conditionally lethal gene. *Gene* **169**, 131-132
17. Hashimoto, Y., Miki, T., Mukae, Ueda, T., and Imoto, T. (1998) Construction of a yeast expression system with positive selection for gene insertion in the absence of a specific phenotype. *Gene* **207**, 167-170
18. Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., Crosa, J.H., and Falkow, S. (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95-113
19. Yanish-Perron, C., Vierra, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: Nucleotide sequence of M13 and pUC19 vectors. *Gene* **33**, 103-119
20. Goodman, R.E. and Spotts, C.R. (1967) Effects of streptomycin deprivation on enzyme synthesis in streptomycin-dependent *Escherichia coli*. *J. Bacteriol.* **94**, 1154-1161
21. Miyanohara, A., Toh-e, A., Nozaki, C., Hamada, F., Ohtomo, N., and Matsubara, K. (1983) Expression of hepatitis B surface antigen gene in yeast. *Proc. Natl. Acad. Sci. USA* **80**, 1-5
22. Maeda, Y., Ueda, T., and Imoto, T. (1998) Effective renaturation of Met-1 lysozyme expressed in *Escherichia coli* as inclusion bodies. *Protein Pept. Lett.* **2**, 67-74
23. Kunkel, T.A., Roberts, J.D., and Zakour, R.A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **254**, 367-382
24. Hinnen, A., Hicks, J.B., and Fink, G.R. (1978) Transformation of yeast. *Proc. Natl. Acad. Sci. USA* **75**, 1929-1933
25. Horwitz, B.H. and Dimaio, D. (1990) Saturation mutagenesis using mixed oligonucleotides and M13 templates containing uracil. *Methods Enzymol.* **185**, 599-611
26. Derbyshire, K.M., Salvo, J.J., and Grindly, N.D.F. (1986) A simple and efficient procedure for saturation mutagenesis using mixed oligodeoxynucleotides. *Gene* **46**, 145
27. Buchner, J. and Rudolph, R. (1991) Renaturation, purification and characterization of recombinant Fab-fragments produced in *Escherichia coli*. *Bio/Technology* **10**, 1289-1295
28. Cryer, D.R., Eccleshal, R., and Marmur, J. (1975) Isolation of yeast DNA. *Methods Cell Biol.* **12**, 39-44
29. Davis, R.W., Thomas, M., Cameron, J., St. John, T.P., Scherer, S., and Padgett, R.A. (1980) Rapid DNA isolations for enzymatic and hybridization analysis. *Methods Enzymol.* **65**, 404-411
30. Imoto, T., Yamada, H., Yasukochi, T., Yamada, E., Ito, Y., Ueda, T., Nagatani, H., Miki, T., and Horiuchi, T. (1987) Point mutation of alanine (31) to valine prohibits the folding of reduced lysozyme by sulfhydryl-disulfide interchange. *Protein Eng.* **1**, 333-338
31. Mine, S., Ueda, T., Hashimoto, Y., and Imoto, T. (1997) Improvement of the refolding yield and solubility of hen egg-white lysozyme by altering the Met residue attached to its N-terminus to Ser. *Protein Eng.* **10**, 1333-1338
32. Ueda, T., Yamada, H., Aoki, H., and Imoto, T. (1990) Effect of chemical modifications of tryptophan residues on the folding of reduced hen egg-white lysozyme. *J. Biochem.* **108**, 886-892
33. Verhamme, I.M., Van, D.G., and Lauwers, A.R. (1988) Ionic-strength-dependent substrate inhibition of the lysis of *Micrococcus luteus* by hen egg-white lysozyme. *Eur. J. Biochem.* **172**, 615-620
34. Hutchinson III, C.A., Nordeen, S.K., Vogt, K., and Edgell, M.H. (1986) A complete library of point substitution mutations in the glucocorticoid response element of mouse mammary tumor virus. *Proc. Natl. Acad. Sci. USA* **83**, 710-714
35. Gething, M.J. and Samstook, J. (1990) Transport and assembly processes in the endoplasmic reticulum. *Semin. Cell Biol.* **1**, 65-72
36. Hammond, C. and Helenius, A. (1995) Quality control in the secretory pathway. *Curr. Opin. Cell Biol.* **7**, 523-529
37. Bonifacino, J.S. and Lippincott-Schwartz, J. (1991) Degradation of proteins within the endoplasmic reticulum. *Curr. Opin. Cell Biol.* **3**, 592-600
38. Cleland, J.L. and Wang, D.I.C. (1990) Refolding and aggregation of bovine carbonic anhydrase B: quasi-elastic light scattering analysis. *Biochemistry* **29**, 11072-11079
39. Tanford, C. (1970) Protein denaturation. C. Theoretical models for the mechanism of denaturation. *Adv. Protein Chem.* **24**, 1-95
40. Back, J.F., Oakenfull, D., and Smith, M.B. (1979) Increased thermal stability of proteins in the presence of sugars and polyols. *Biochemistry* **18**, 5191-5196
41. Gekko, K. and Timasheff, S.N. (1981) Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. *Biochemistry* **20**, 4667-4676
42. Gekko, K. and Timasheff, S.N. (1981) Thermodynamic and kinetic examination of protein stabilization by glycerol. *Biochemistry* **20**, 4677-4686
43. Sawano, H., Koumoto, Y., Ohta, K., Sasaki, Y., Segawa, S., and Tachibana, H. (1992) Efficient *in vitro* folding of the three-disulfide derivatives of hen lysozyme in the presence of glycerol. *FEBS Lett.* **303**, 11-14
44. Tachibana, H., Ohta, K., Sawano, H., Koumoto, Y., and Segawa, S. (1994) Relationship between the optimal temperature for oxidative refolding and the thermal stability of refolded state of hen lysozyme three-disulfide derivatives. *Biochemistry* **33**, 15008-15016
45. Hol, W.G., van, Duijunen, P.T., and Berendsen, H.J. (1978) The alpha-helix dipole and the properties of proteins. *Nature* **273**, 443-445
46. Richardson, J.S. and Richardson, D.C. (1988) Amino acid preferences for specific locations at the ends of alpha helices. *Science* **240**, 1648-1652
47. Abe, Y., Ueda, T., Iwashita, H., Hashimoto, Y., Motoshima, H., Tanaka, Y., and Imoto, T. (1995) Effect of salt concentration on the p$K_a$ of acidic residues in lysozyme. *J. Biochem.* **118**, 946-952
48. Motoshima, H., Mine, S., Masumoto, K., Abe, Y., Iwashita, H., Hashimoto, Y., Chijiiwa, Y., Ueda, T., and Imoto, T. (1997) Analysis of the stabilization of hen lysozyme by helix macrodipole and charged side chain interaction. *J. Biochem.* **121**, 1076-1081

Downloaded from http://jb.oxfordjournals.org/ at Changhua Christian Hospital on October 1, 2012