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Efficient in vitro folding of the three-disulfide derivatives of hen lysozyme in the presence of glycerol

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Four derivatives of hen lysozyme, each lacking one native disulfide bond of the four in authentic lysozyme, were produced in *Escherichia coli* by expressing synthetic mutant genes. In the reoxidation reaction of the reduced derivatives purified from inclusion bodies, the addition of glycerol significantly enhanced the efficiency of folding and 'correct' disulfide bond formation. This enabled simple chromatographical purification of refolded materials. Purified 3SS-derivatives all showed lytic activities and secondary structures comparable to authentic lysozyme, which directly showed that none of the four native disulfide bonds is a prerequisite for 'correct' in vitro folding.

Protein folding; Disulfide bond; Lysozyme; Renaturation; Glycerol

1. INTRODUCTION

The contribution of individual disulfide bonds to the folding and stability of proteins which have multiple disulfide bonds has only been examined in a few proteins [1–5]. The detailed pathway of folding has been presented only for BPTI [1]. We have synthesized, and expressed in *E. coli*, the genes for all the four 3SS-derivatives of hen lysozymes, Δ1, Δ2, Δ3 and Δ4, in which one of the four native disulfide bonds, Cys^{6}–Cys^{127}, Cys^{30}–Cys^{115}, Cys^{64}–Cys^{80} and Cys^{76}–Cys^{94} (hereafter referred to as disulfide bonds 1, 2, 3 and 4, respectively), was opened by substitution of Ser for Cys residues (Fig. 1a,b). We show efficient conditions for the folding and 'correct' disulfide bond formation of the reduced 3SS-derivatives, and the enzymatic acitivity and secondary structure of the refolded 3SS-derivatives.

2. MATERIALS AND METHODS

2.1. *Materials*

A direct expression vector, pYK1, has been described [6]. *E. coli* strain AD18 (*Δ (lac-proAB), lon*-100, *tsx*::Tn*5*/F′[*lacI*^{q}, *lacZΔM15*, *lacY*$^{+}$, *proA*$^{+}$, *proB*$^{+}$]) was used for expression.

2.2. *DNA synthesis*

Oligodeoxyribonucleotides were synthsized on an Applied Biosystems 381A DNA synthesizer at the Research Center for Molecular Biology, Kobe University. Two sets, coding and non-coding, of 14 oligomers constitute the total gene (Fig. 1c). For two sets of 8 oligomers among them which contained the codon or anticodon sequence for a Cys residue, oligomers with the codon (or anticodon) sequence of Ser substituted for Cys were also synthesized. Manipulations of DNAs were carried out as described [7]. The 5′-end of the gene had a *Hin*dIII cohesive sequence. It also had a *Dra*I site (TTT′AAA) incorporated to facilitate excision of the gene from the cloning plasmid with the codon AAA, coding for the N-terminal lysine residue, exposed as a blunt end for its insertion to pYK1. The 3′-end contained a TAA termination codon and a *Bam*HI cohesive sequence.

Abbreviations: 3SS-, three disulfide bond-.

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2.3. *Expression of mutant genes and purification of the polypeptides*

Expression was carried out as described [8] except that we used *E. coli* strain AD18 and LB-medium containing ampicillin (25 μg/ml) and kanamycin (50 μg/ml). Harvested cells were disrupted by sonication and inclusion bodies were prepared as described [9]. They were solubilized in 8 M urea and 50 mM DTT, and reduced 3SS-derivatives were purified with cation-exchange (Mono-S) and gel-permeation (Sephadex G-75) chromatographies, freeze-dried and stored frozen under nitrogen.

2.4. *Peptide analysis*

Reduced and carboxamidomethylated [10] protein in 100 mM Tris-HCl, pH 8.0, was digested with TPCK-trypsin (E:S=1:100 by weight) at 30°C for 3 h or more. Tryptic peptides were separated with reversed-phase HPLC. The amino acid composition of each peptide was determined as described [11].

2.5. *Reoxidation of reduced protein*

Reoxidation and formation of disulfide bonds were carried out essentially as described [12], in 100 mM Tris-acetate, 1 mM EDTA, pH 7.8, 6 mM GSH and 0.6 mM GSSG, at the protein concentration of 3.3 μM, with the modification that the indicated amounts of glycerol were added and the indicated temperature was used. After 2 h of reoxidation the remaining (if any) thiol groups were carboxamidomethylated, and the reoxidation reaction was terminated by acidifying to below pH 5. The oxidized protein was purified by RPHPLC on a TSK TMS-250 column (4.6 mm × 7.5 cm; Tosoh) with a linear gradient of acetonitrile from 5 to 35% in 0.05% TFA.

2.6. *Other methods*

Lytic activity against *Micrococcus luteus* cells was measured as described [12]. Concentrations of the 3SS-derivatives were estimated as described [13]. CD spectra were measured using a J-600 spectropolarimeter (Japan Spectroscopic Co.) equipped with a thermostatically controlled cell holder.

3. RESULTS

We employed total gene synthesis because we also planned to synthesize 2SS- and 1SS-derivatives as well as fragments of hen lysozyme for future studies. The genes (Fig. 1) were synthesized in two steps of ligation. The recovered full-length gene was ligated to *Hin*dIII- and *Bam*HI-digested pUC18, with which *E. coli* JM109 was transformed, and the genes for Δ1 through Δ4 were cloned. Their nucleotide sequences were confirmed. Each gene was then excised by digestion with *Dra*I and *Bam*HI, gel-purified, and ligated to pYK1 which had been digested with *Nco*I, filled-in and digested with *Bam*HI. The expressed products were found mostly in inclusion bodies. The yield of the purified polypeptides were 1.0 to 6.2 mg per liter of culture. Tryptic peptide mapping of the reduced and carboxamidomethylated polypeptides confirmed correct substitution of Ser for Cys residues in all the four derivatives.

Reoxidation and formation of disulfide bonds under the conditions previously described for authentic lysozyme [12] gave materials with a low lytic activity (Table I) and a broad elution profile on RPHPLC (Fig. 2), probably due to a low stability of the folded state of these derivatives. When we lowered the temperature for the reoxidation reaction to 15°C, the activity values for the reoxidized materials increased and their elution profiles became sharp (not shown). Further decrease in reoxidation temperature, however, gave a lower activity and a broad profile again. Next, we added glycerol, which has been known to increase the stability of the native state of proteins [14] to the reoxidation solution, and found that the elution profile of the reoxidized materials became very sharp (Fig. 2). The elution time of the main peak for Δ1 through Δ4 nearly coincided with that for authentic lysozyme. Rechromatography of the main-peak fraction with RPHPLC or cation-exchange HPLC gave an apparently single peak for all the four derivatives (not shown). The tryptic-peptide maps for the reoxidized and purified derivatives, and the results of the amino acid analysis for the peptide peaks which newly appeared on the reoxidation reaction, were consistent with the formation of the three 'correct' disulfide bonds in each of the four derivatives (not shown).

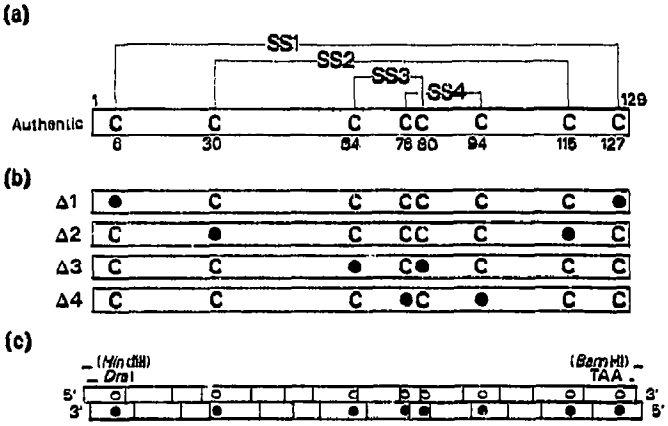

Fig. 1. (a) Eight cysteine residues ('C') and four disulfide bridges (SS1 to SS4) in hen lysozyme. (b) The four 3SS-derivatives. Filled circles represent substitutions of Ser for Cys residues. (c) Nucleotide regions covered by the 28 oligomers (rectangular blocks) which make up a synthetic gene. Open and filled circles represent the codon and anticodon sequences for Cys residues.

The activity of the purified 3SS-derivatives was comparable to that of authentic lysozyme (Table I). Circular dichroism spectra (Fig. 3) showed that they had secondary structures comparable to that of authentic lysozyme. The mean residue ellipticity values were independent of the protein concentration (from 4 to 20 μM) indicating that the observed secondary structure was intramolecularly formed. Their spectral shapes were different from each other. In particular, Δ2 showed a marked difference from the rest.

4. DISCUSSION

We have shown that none of the four disulfide bridges of native lysozyme is obligatory for correct in vitro

Table I

Lytic activities of the 3SS-derivatives of lysozyme

3SS-species	Recombinant synthesized in *E. coli*[a]		Partial oxidation or reduction	Recombinant secreted from yeast [16]
	Refolded at 37°C, no glycerol[b]	Refolded at 15°C, in 20% glycerol[c]		
Δ1	9 (%)[d]	68 ± 2[e] (%)[d]	40–50 [15], 58 [18] (%)[d]	ND[f] (%)[g]
Δ2	10 (%)[d]	76 ± 7 (%)[d]	ND	28
Δ3	13 (%)[d]	88 ± 4 (%)[d]	40–50 [15]	23
Δ4	19 (%)[d]	142 ± 11 (%)[d]	40–50 [15]	94

[a]This study, [b]reoxidized materials, not purified, [c]purified main peak fraction, [d]values relative to authentic hen lysozyme, [e]mean and SD for three measurements, [f]not determined, [g]values relative to authentic human lysozyme.

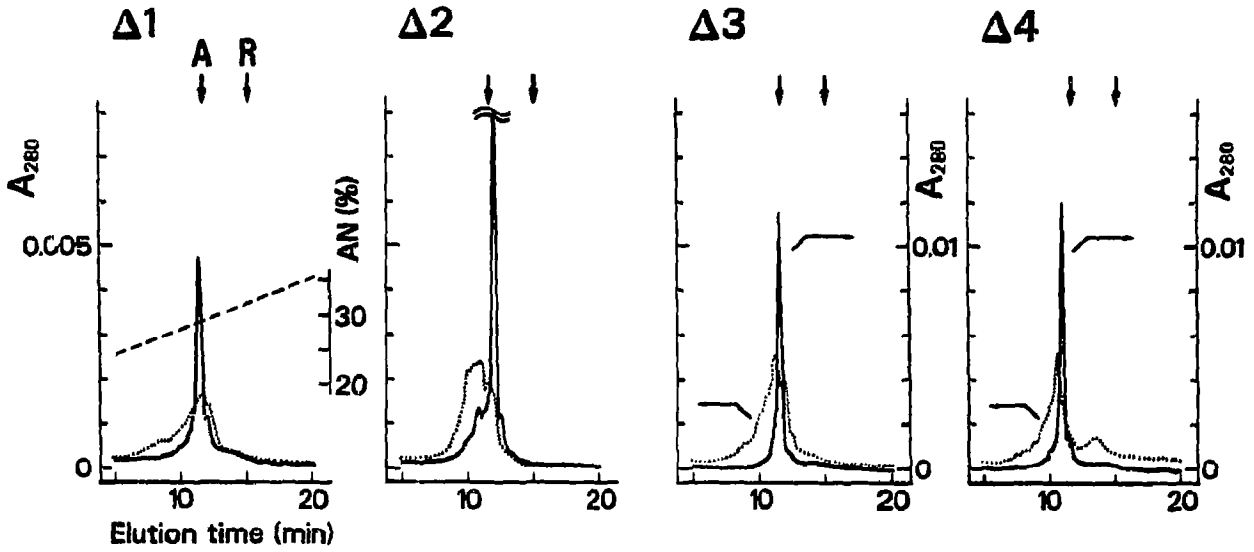

Fig. 2. Elution profiles of the 3SS-derivatives reoxidized at 37°C without glycerol (dotted lines) and those at 15°C with 20% glycerol (full lines). The gradients in acetonitrile are in broken lines. The amount of protein reoxidized was about 4 μg, the filtrate of which was subjected to RPHPLC. A and R indicate the elution positions of authentic and reduced lysozymes, respectively.

folding of hen lysozyme to enzymatically active forms. The same conclusion has been drawn [4] using the materials obtained through reoxidation of reduced and partially, randomly, carboxymethylated hen lysozyme. Our approach was more direct, and since all the four 3SS-derivatives were obtained in significant amounts due to efficient folding in the presence of glycerol, we could also examine their secondary structures, which turned out not to be identical. Previously, except for the 3SS-derivative corresponding to Δ1, it was difficult to obtain other 3SS-derivatives in sufficient amounts. In particular, the derivative corresponding to Δ2 could not be obtained [15]. In recent studies on human lysozyme, although the derivative corresponding to Δ4 was synthesized in large amounts, the derivative corresponding to Δ1 was not secreted in the yeast expression system, and those corresponding to Δ2 and Δ3 were secreted inefficiently [16,17]. Detailed analyses of the structural differences among the 3SS-derivatives constructed here will help to understand the interactions inducing and stabilizing intramolecular structures in the folding of hen lysozyme.

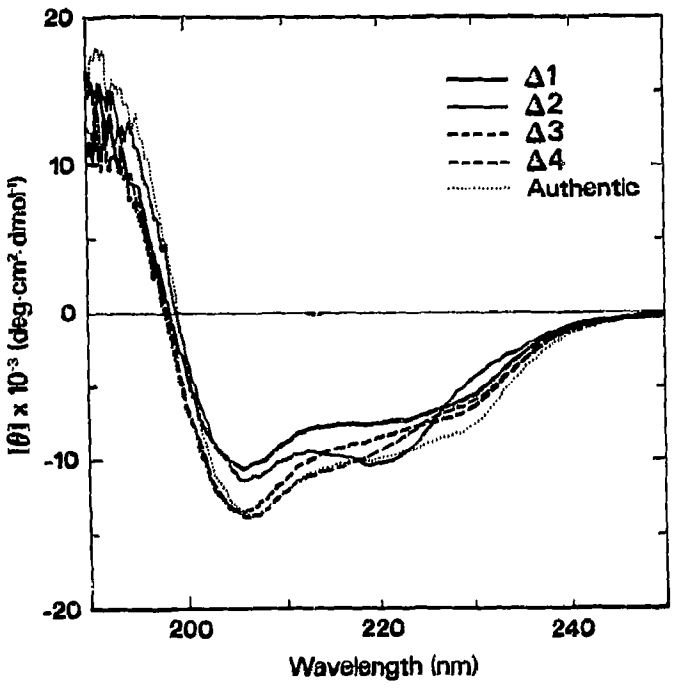

Fig. 3. Circular dichroism spectra for purified Δ1 (thick line), Δ2 (thin line), Δ3 (thick broken line), Δ4 (thin broken line) and authentic lysozyme (dotted line) in 20 mM phosphate adjusted to pH 3.9 with sodium hydroxide and at 25°C. Protein concentrations were 4.0, 3.4, 3.6, 3.7 and 3.5 μM, respectively.

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