Relationship between the Optimal Temperature for Oxidative Refolding and the Thermal Stability of Refolded State of Hen Lysozyme Three-Disulfide Derivatives†

Hideki Tachibana,*,‡,§ Katsuyuki Ohta,‡,⊥ Hiroshi Sawano,§ Yasuko Koumoto,∥,# and Shin-ichi Segawa∥

Department of Biology, Faculty of Science, and Graduate School of Science and Technology, Kobe University, Rokkodai, Nada-ku, Kobe 657, Japan, and Department of Physics, School of Science, Kwansei Gakuin University, Nishinomiya 662, Japan

Received March 31, 1994; Revised Manuscript Received October 10, 1994⊗

ABSTRACT: The temperature dependence of the efficiency of oxidative refolding was examined for hen lysozyme three-disulfide derivatives produced in *Escherichia coli*. Each derivative was designed to lack one of the four disulfide bridges in authentic lysozyme: Δ1 (Cys6→Ser, Cys127→Ser), Δ2 (Cys30→Ser, Cys115→Ser), Δ3 (Cys64→Ser, Cys80→Ser), Δ4 (Cys76→Ser, Cys94→Ser), Δ2Ala (Cys30→Ala, Cys115→Ala), and Δ4Ala (Cys76→Ala, Cys94→Ala). The optimal refolding temperature was lowest for Δ1 (19 °C) and highest for Δ4A1a (30 °C). The chromatographically purified, completely refolded three-disulfide species were not stable above the optimal refolding temperature in the presence of glutathione. The stability of each of them was determined from the far-UV CD thermal denaturation measurement at pH 3.9 in the absence of glutathione, where the denaturation was reversible. The transition temperature was lowest for Δ1 and highest for Δ4Ala. Precise values of difference in the transition temperature among the three-disulfide derivatives were found to correlate with those in the optimal refolding temperature. Next, the effect of glycerol, which has been shown to increase the refolding efficiency [Sawano et al. (1992) *FEBS Lett. 303*, 11−14], was examined for Δ1 in detail. The optimal temperature for refolding increased by 3−4 °C with the increase in glycerol concentration by 10%. The amount of increase in the optimal refolding temperature was nearly equal to the amount of the increase in thermal stability in the presence of glycerol of refolded and purified Δ1. Taken together, there exists a parallel relationship for the three-disulfide derivatives between the optimal refolding temperature and the thermal stability of the correctly refolded state. The observation provides the basis for the optimization of the refolding temperature of engineered proteins of low stability.

Expression of recombinant, foreign genes in *Escherichia coli* often leads to the formation of inclusion bodies in which recombinant proteins are produced as insoluble aggregates (Mitraki & King, 1989). The polypeptides solubilized and purified from inclusion bodies under denaturing and reducing conditions should be renatured into functional proteins of defined tertiary structure. It is therefore needed to develop efficient methods of renaturation. For the proteins which contain disulfide bridges, the refolding conditions which utilize reduced and oxidized forms of glutathione (Saxena & Wetlaufer, 1970) are widely used. The detailed reaction conditions, however, such as temperature, pH, salt concentration, and redox potential have to be optimized empirically.

Hen lysozyme contains four disulfide bridges. In spite of a number of studies on the folding reaction of lysozyme, it has not fully been clarified how these bridges are involved in the reaction process (Ristow & Wetlaufer, 1973; Anderson & Wetlaufer, 1976; Acharya & Taniuchi, 1976, 1977, 1982; Taniyama et al., 1988; Radford et al., 1991). To study the role of each bridge in the folding as well as in the stabilization of this protein, we have undertaken the construction of all the four molecular species of hen lysozyme three-disulfide derivatives in which Cys residues that form a disulfide bridge in authentic lysozyme were replaced by Ser: Δ1[1] (Cys6→Ser, Cys 127→Ser), Δ2 (Cys30→Ser, Cys115→Ser), Δ3 (Cys64→Ser, Cys80→Ser), and Δ4 (Cys76→Ser, Cys94→Ser) (Sawano et al., 1992). The 3SS derivatives purified from inclusion bodies under denaturing and reducing conditions did not efficiently refold under the conditions which have been reported by Saxena and Wetlaufer (1970) to be optimal for the oxidative refolding (i.e., regeneration of correct disulfide bridges and of nearly full enzymatic activity) of authentic lysozyme. We showed, however, that they refolded at low temperatures and/or in the presence of glycerol.

In this study, to examine the factors which influence the refolding efficiency, we determined the optimal refolding temperatures for the four 3SS derivatives as well as two

† This work was partly supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture, Japan, and by a grant to S.S. from Hyogo Science and Technology Association.

* Author to whom correspondence should be addressed.

‡ Department of Biology, Kobe University.

§ Graduate School of Science and Technology, Kobe University.

⊥ Present address: Chemical Process Research and Development, Production Department, Shionogi & Co. Ltd., 1-3, Kuise Terajima 2-chome, Amagasaki 660, Japan.

∥ Department of Physics, Kwansei Gakuin University.

Present address: Department of Cell Biology, National Institute for Basic Biology, Okazaki 444, Japan.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1994.

[1] Abbreviations: 3SS, three-disulfide; Δ1, a 3SS derivative of hen lysozyme in which Cys residues 6 and 127 are replaced by Ser residues; (for other 3SS derivatives, the amino acid substitutions are as follows: Δ2 (Cys30→Ser, Cys115→Ser), Δ3 (Cys64→Ser, Cys80→Ser), Δ4 (Cys76→Ser, Cys94→Ser), Δ2A1a (Cys30→Ala, Cys115→Ala), and Δ4Ala (Cys76→Ala, Cys94→Ala)); T_{opt}, optimal temperature for the oxidative refolding from reduced state; T_m, midpoint temperature for the unfolding reaction (without the opening of disulfide bridges); RPHPLC, reversed-phase high-performance liquid chromatography; GSH, reduced glutathione; GSSG, oxidized glutathione; RNase A, bovine pancreatic ribonuclease A; α-LA, α-lactalbumin.

0006-2960/94/0433-15008$04.50/0

Cys→Ala 3SS derivatives: Δ2Ala (Cys30→Ala, Cys115→Ala) and Δ4Ala (Cys76→Ala, Cys94→Ala). The optimal temperatures were related with the thermal stability of the derivatives correctly refolded.

MATERIALS AND METHODS

Hen Lysozyme 3SS Derivatives. Construction of the synthetic genes for Δ1−Δ4 and their direct expression in *E. coli*, based on the reported method (Miki et al., 1987), have been described (Sawano et al., 1992). The genes for Δ2Ala and Δ4Ala were constructed by using the synthetic oligonucleotides, which contained the codons for Ala residues in place of the respective Cys residues. The nucleotide sequence of each recombinant gene cloned in plasmid was confirmed. The genes were then incorporated to the direct expression vector pYK1 (Tachibana et al., 1990), and expressed in a similar way as for Δ1−Δ4.

Purification of the polypeptides under reducing (and denaturing) conditions was carried out as follows. Harvested cells (from 5.4-L culture) were disrupted by sonication in 200 mL of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride. Inclusion bodies were prepared as described (Nagai et al., 1987). They were then solubilized in 24 mL of 8 M urea, 10 mM sodium phosphate, 1 mM EDTA, and 50 mM DTT, pH 7.9, at 37 °C for 2 h. After the pH was lowered to 5.0 with acetic acid, the solution was filtered through a 0.8-μm filter and subjected to cation-exchange chromatography on a SE column (Productive, bps Separations Ltd.) which had been equilibrated with 8 M urea, 10 mM sodium acetate, 1 mM EDTA, and 10 mM DTT, pH 5.0, and eluted with step gradients in NaCl concentration. Fractions containing polypeptides of 14 kDa, as monitored with SDS−PAGE, were subjected to gel permeation chromatography on a Sephadex G-75 column (2.5 × 50 cm), and reduced polypeptides were eluted with 0.1 M acetic acid, freeze-dried, and stored frozen under nitrogen. The concentration of the purified, reduced 3SS derivatives were estimated by using $A_{280} = 2.64$ for 1 mg/mL protein, which was deduced based on the content of tryptophan and tyrosine residues (Gill & von Hippel, 1989). The yield of purified polypeptide was 7−10 mg/L of culture.

The substitutions of Ser or Ala for Cys residues in the purified, reduced 3SS derivatives were confirmed by tryptic peptide mapping. Briefly, the polypeptides were carboxamidomethylated (Hirs, 1967) and digested with *N*-tosyl-L-phenylalanyl chloromethyl ketone-treated trypsin (Seikagaku Kogyo) (E:S = 1:100 in weight) in 100 mM Tris-HCl, pH 8.0, at 30 °C for 3 h. The tryptic peptides were separated with reversed-phase HPLC on TSK-ODS120T column (7.8 mm × 30 cm; Tosoh) with a 50-min gradient of acetonitrile from 1 to 50% in 0.1% TFA at 40 °C. The polypeptides in each peak were hydrolyzed with 6 N HCl containing 0.01% phenol, and the amino acids were derivatized with phenyl isothiocyanate (sequencing-grade, Wako Pure Chemical Industries, Ltd.) as described by Heinrikson and Meredith (1984). Phenylthiocarbamyl amino acids were separated and quantitated with reversed-phase chromatography on a TSK-ODS80TM column (4.6 mm × 25 cm; Tosoh) with a 20-min gradient from 3% acetonitrile, 50 mM sodium phosphate (pH 7.0), and 100 mM sodium perchlorate to 33% acetonitrile, 10 mM sodium phosphate (pH 7.0), and 20 mM sodium perchlorate.

Reoxidation of Reduced Protein and Purification of Refolded Protein. Reoxidation and formation of disulfide bonds were carried out in 100 mM Tris−acetate, 1 mM EDTA, pH 7.8, 6 mM GSH, and 0.6 mM GSSG as described (Saxena & Wetlaufer, 1970), except that various temperatures and, when specified, glycerol concentration (v/v %) were used. The protein concentration was 3.3 μM. For the measurement of the refolding efficiency at each temperature and glycerol concentration, 6−12 μg of protein was used. The temperature dependence of the pH of the refolding buffer was not corrected for. The shift in pH value due to the presence of glycerol was not more than 0.2. Before being mixed to start refolding, both the refolding buffer and the reduced protein in 0.1 N acetic acid were equilibrated at the reaction temperature. The refolding reaction was stopped by adding acetic acid to bring the solution pH below 5, and the solution was passed to activity measurement.

As the control in the reoxidation experiment, reduced authentic lysozyme was also prepared. Hen egg-white lysozyme (6× crystallized, Seikagaku Kogyo) was reduced in 0.2 M Tris-HCl, 1 mM EDTA, 8 M urea, pH 8.0, and 50-fold molar excess of DTT over the content of disulfide in the protein at 40 °C for 3 h; subjected to gel permeation chromatography in 0.1 N acetic acid on Sephadex G25; and freeze-dried.

To know the extent of conformational heterogeneity in the reoxidized proteins, the reoxidized protein solution was subjected to reversed-phase HPLC after being acidified below pH 5 or after the remaining, if any, thiol groups were carboxamidomethylated on a TSK TMS-250 column (4.6 mm × 7.5 cm, Tosoh) at 40 °C with a linear gradient of acetonitrile from 28 to 40% in 0.05% TFA. Native and reduced authentic lysozymes were used as references for elution position.

Reduced 3SS derivatives were reoxidized in a larger scale (300−900 μg) at the temperature and the glycerol concentration, which were determined to be optimal in the preceding experiments. The main peak fraction on the reversed-phase HPLC described above was recovered, freeze-dried, and used for stability measurement. The protein in the main peak fraction eluted as a single peak when rechromatographed on reversed-phase or cation-exchange (Asahipak ES-502C, 7.6 mm i.d. × 100 mm, Asahi Kasei Kogyo) HPLC.

Activity Measurement. The efficiency of refolding was expressed by the lytic activity observed for the reoxidized material. A portion of the solution that contained 3.3 μg of the reoxidized 3SS derivative protein was mixed with the solution of *Micrococcal luteus* cells (Seikagaku Kogyo) in 50 mM phosphate buffer, pH 6.20, which had been equilibrated at 25 °C, to the total volume of 1.10 mL and the final cellular concentration of 0.35 mg/mL. The time derivative of the 660-nm absorbance decreases 30 s after the mixing was taken as the lytic activity value and shown as a value relative to that for an equal amount of authentic lysozyme. When necessary, the reaction scale was increased for a better reproducibility. The relative activity values varied from one experiment to another by less than 4% (Δ1, Δ2, Δ3) or 7% (Δ2Ala, Δ4, Δ4Ala). (Here, 100% corresponds to the activity of authentic lysozyme.) The resultant deviation in each T_{opt} value was not more than ±2 °C. The presence of glycerol, GSSG, or GSH to the amount which was expected to be carried over to the assay solution did not decrease or

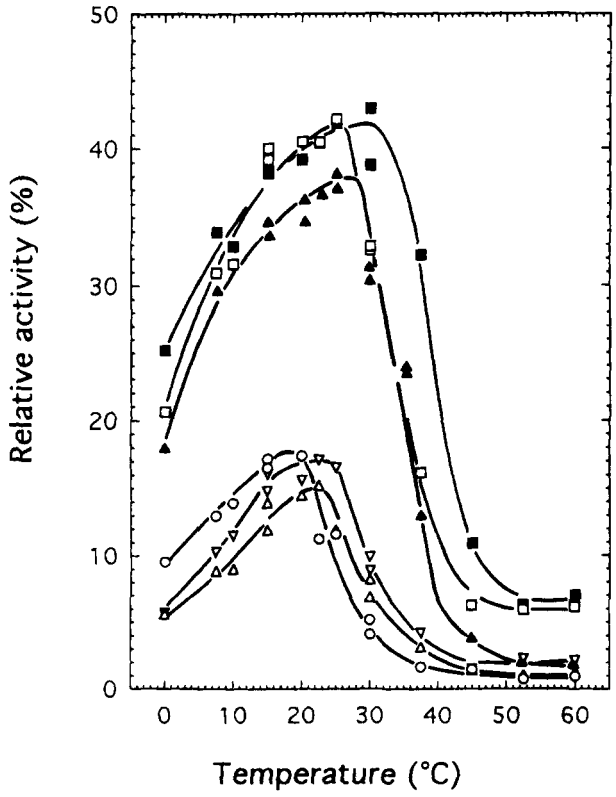

FIGURE 1: Lytic activity of the solution of each 3SS derivative refolded at various temperatures. Symbols and T_{opt} values are (○) Δ1, 19 °C; (△) Δ2, 22 °C; (▲) Δ2Ala, 27 °C; (▽) Δ3, 23.5 °C; (□) Δ4, 25 °C; (■) Δ4Ala, 30 °C.

increase in a control experiment the activity of authentic lysozyme.

Stability Measurement. The stability of chromatographically purified, completely refolded 3SS derivatives in the presence of reducing reagent and at a temperature higher than T_{opt} was examined as follows: The protein solution in 0.1 N acetic acid and the buffer containing Tris−acetate, EDTA, and reduced and oxidized glutathione both equilibrated at the temperature were mixed, resulting in the same solution conditions and protein concentration as used in the refolding experiment. After various intervals, the solution was acidified to pH < 5, filtered through a 0.2-μm filter, and analyzed with RPHPLC on a TSK TMS-250 column as described above. As a control, the buffer lacking the thiol reagents was also used.

CD thermal transition curves were obtained from the far-UV region CD spectra measured at various temperatures using a J-600 spectropolarimeter (Japan Spectroscopic Co.) equipped with a thermostatically controlled cell holder. The solvent was 20 mM phosphate, with or without glycerol, adjusted to pH 3.90 (at room temperature) with sodium hydroxide. Protein concentration was 3.0−6.4 μM. The temperature of the solution was directly measured using a thermistor thermometer.

RESULTS

Optimal Temperatures for the Refolding Reaction of 3SS Derivatives. The temperature dependence of the refolding reaction for the 3SS derivatives is shown in Figure 1. The ordinate shows the bacteriolytic activity of the reoxidized protein solution, in which a mixture of refolded proteins of various tertiary structure or disulfide pairing was expected to be contained. The temperature dependence for each derivative was qualitatively similar to the typical temperature profile of the enzyme activity: gradual increase to the maximal level and steep decrease with the increase in temperature. The optimal refolding temperatures were 19, 22, 27, 23.5, 25, and 30 °C for Δ1, Δ2, Δ2Ala, Δ3, Δ4, and Δ4Ala, respectively. Among the Cys→Ser derivatives, the result was Δ1 < Δ2 < Δ3 < Δ4 in the increasing order of T_{opt}. It was about 5 °C higher for the Cys→Ala derivative than for its counterpart Cys→Ser derivative. The observed bacteriolytic activity was much higher for Δ2Ala, Δ4, and Δ4Ala than for Δ1, Δ2, and Δ3. Under the same refolding conditions as above, T_{opt} for reduced authentic lysozyme was about 35 °C, and the relative bacteriolytic activity of its refolded solution amounted to 90% (not shown) in agreement with a previous paper (Saxena & Wetlaufer, 1970). These data were obtained in the reoxidation experiments of 50-min incubation time. Although kinetic experiments showed that in this time interval the reaction did not reach equilibrium for lower reaction temperatures, the experiments employing an incubation time of 200 min showed that qualitative as well as quantitative features described above were conserved: the newly estimated T_{opt}'s agreed with the original one within ±1 °C (not shown).

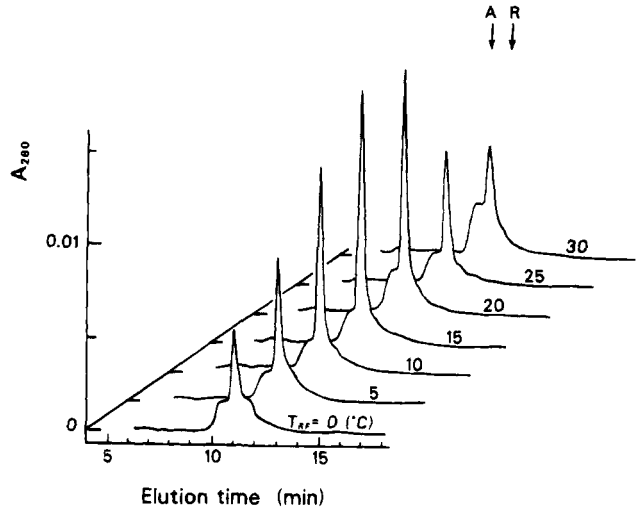

FIGURE 2: Elution profiles of the refolded Δ1 on RPHPLC. Each profile is labeled with the temperature, T_{RF}, at which the refolding reaction was carried out. 8.2 μg of reduced Δ1 was refolded, filtered through a 0.2-μm filter, and subjected to RPHPLC as described in Materials and Methods. Arrows A and R indicate the elution positions for authentic and reduced lysozymes, respectively.

To examine the conformational heterogeneity among the reoxidized proteins, the reoxidized solution was subjected to reversed-phase HPLC. The result for Δ1 is shown in Figure 2. A peak on a broad background was observed for each refolding condition of different temperature. The elution time for the peak was common among the profiles shown and nearly coincided with that of authentic lysozyme (position "A" in the figure). There existed a correlation between the height of the peak and the apparent lytic activity value shown for various temperatures in Figure 1. A similar result was obtained for other 3SS derivatives (not shown). Each temperature profile in Figure 1 therefore can be taken as to approximately represent the change with temperature in the fraction of correctly refolded 3SS derivative. (For a comparison of the fractions of correctly refolded molecule

between the profiles, however, it is necessary to take the specific activity of each 3SS derivative into consideration.) This point was supported by the following observations: First, the refolded 3SS derivatives isolated in a large scale from the peak fraction showed the far-UV CD spectra that indicated the presence of secondary structure of nearly the same amount as authentic lysozyme [the spectra of the Cys→Ser derivatives have been reported (Sawano et al., 1992); the spectra of Δ2Ala and Δ4Ala had the same characteristics as those for their respective, counterpart Cys→Ser derivatives (not shown)]. Second, the tryptic map of each of the purified, refolded derivatives was consistent with the formation of each set of the three, out of four, native disulfide bridges in them (not shown). Third, the purified refolded derivatives showed lytic activities which were comparable (60−140%) to authentic lysozyme (Sawano et al., 1992, for the Cys→Ser derivatives; the relative activities at 25 °C for Δ2Ala and Δ4Ala were 72 and 90%, respectively). Furthermore, when we calculated the expected lytic activity of the crude, refolded protein solution by using the estimated amount of the "correctly" refolded protein of the peak fraction in the chromatogram shown in Figure 2 and the activity value of the purified derivatives described above, it nearly agreed with the observed lytic activity value shown in Figure 1.

Thermal Stability of the Refolded and Purified 3SS Derivatives and Its Relationship with T_{opt}. The decrease in the refolding efficiency in the temperature range above T_{opt} is considered to be due to thermal instability of intermediate species and/or the completely refolded species in the folding pathway. We examined the stability of the completely refolded and chromatographically purified Δ1, which was subjected to the same solution conditions as those in the refolding experiment, by monitoring the RPHPLC elution profiles (Figure 3). A reaction temperature of 30 °C was chosen, where the recovered activity in the refolding experiment had been shown to decrease to about 25% of the optimal one (Figure 1). After 90-s incubation, the height of the peak for the completely and correctly refolded Δ1 decreased to 80%, and a broad background, which was indicative of disulfide-reshuffled proteins of nonnative structure, appeared. After 15 min, the peak height decreased to 29%, and after 50 min it decreased to 22%, with the elution profile becoming very similar to the one shown for a refolding temperature (T_{RF}) of 30 °C in Figure 2. The fraction of the intact Δ1 remaining after the 50-min incubation roughly agreed with the above-mentioned fraction of the recovered activity at 30 °C to the optimal one. In a control experiment in which the redox reagents were omitted, the sharp peak was completely conserved after 50-min incubation. Similar results were obtained for the other 3SS derivatives (not shown). The results indicate that the correctly refolded 3SS derivatives are instable at the temperature above T_{opt} in the present conditions used for refolding.

It was difficult with spectrophotometric methods to accurately determine the denaturation temperature of the completely refolded and chromatographically purified 3SS derivatives under the conditions used for refolding, i.e., in the presence of the redox reagents and at the dilute protein concentration. Even in the absence of the redox reagents, the solution of the refolded and purified derivatives showed aggregation at neutral pH regions. Therefore, we carried

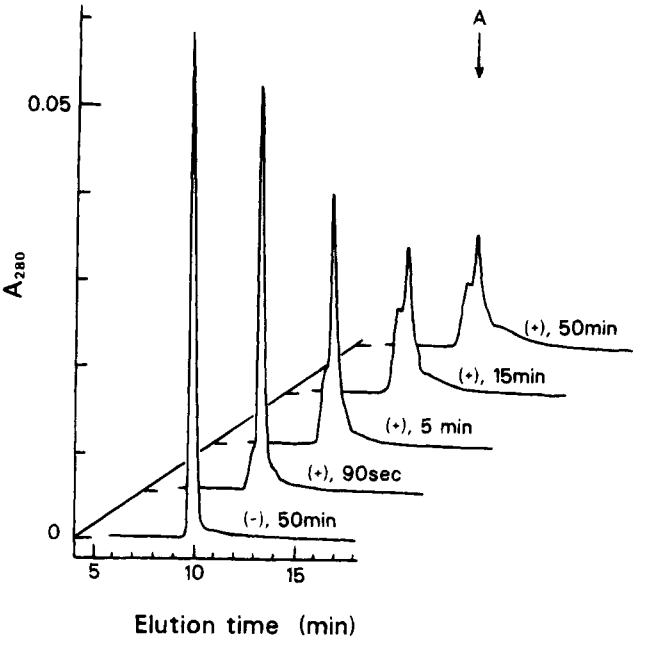

FIGURE 3: Stability of the completely refolded and chromatographically purified Δ1 under the refolding solution conditions and at 30 °C. The label to each RPHPLC elution profile indicates the presence (+) or absence (−) (as a control) of thiol reagents (6 mM GSH and 0.6 mM GSSG) and the incubation time. The amount of protein for each chromatogram was 7.5 μg (including a possible loss during filtration). Arrow A indicates the elution position for authentic lysozyme. It is not the same as the one in Figure 2 due to the aging of the column.

out far-UV CD thermal denaturation measurement at pH 3.9 without the redox reagents, although the denaturation temperature under this condition is expected to be higher than that under the refolding condition. At pH 3.9, reversible transition curves were obtained for all the derivatives (Figure 4). The transition midpoints (T_m) were 39.2, 42.8, 48.2, 45.2, 44.9, and 51.6 °C (±0.6 °C) for Δ1, Δ2, Δ2Ala, Δ3, Δ4, and Δ4Ala, respectively. Among the Cys→Ser derivatives, the result was Δ1 < Δ2 < Δ3 < Δ4 in the increasing order of T_m, and the Cys→Ala derivatives had the T_m which was higher than that of the counterpart Cys→Ser derivative by about 6 °C. Furthermore, the T_m data obtained in our preliminary differential scanning calorimetry measurements (not shown) agreed with these results.

T_{opt} and T_m for each 3SS derivative were plotted in Figure 5. A nearly parallel relationship between the two quantities was evident. The difference between them, which was almost constant among the derivatives, was 20.9 ± 0.7 °C.

Effect of Glycerol on T_{opt} and T_m of Δ1. It has been known that glycerol stabilizes the native state of protein through the mechanism of "preferential hydration" (Gekko & Timasheff, 1981a,b). We have shown that the presence of glycerol increased the refolding efficiency of the Cys→Ser 3SS derivatives (Sawano et al., 1992). To study the effect of glycerol on the temperature dependence of refolding reaction, the efficiency of refolding at various glycerol concentration and temperatures was examined for Δ1 (Figure 6). With the increase in glycerol concentration up to 30%, the temperature−activity profile shifted to the higher temperature side with a concomitant increase in the maximal activity at T_{opt}. With a further increase in glycerol concentration, the profile further shifted to the higher temperature side, but the maximal activity did not increase. The optimal

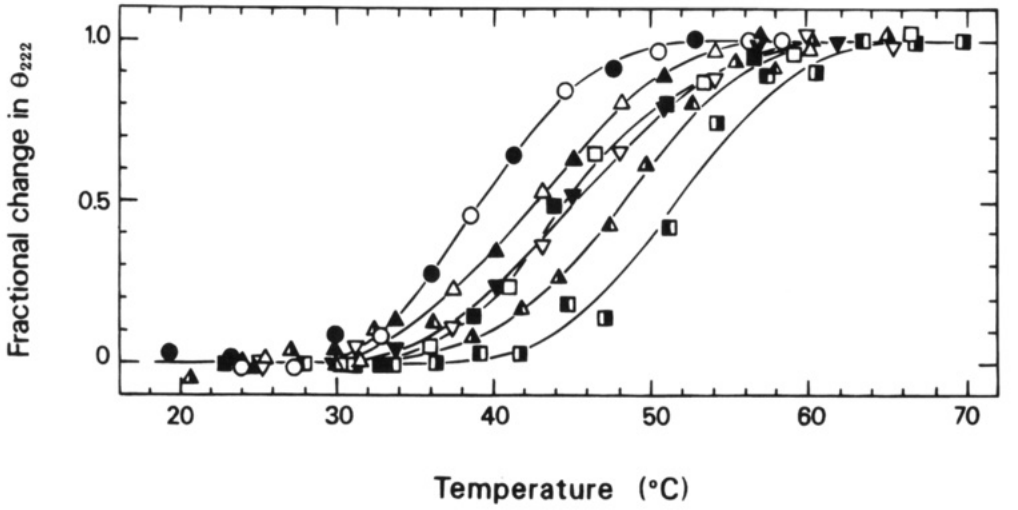

FIGURE 4: Thermal denaturation−renaturation transition curves for the 3SS derivatives. The ordinate shows fractional changes in the mean residue ellipticity at 222 nm. Symbols are as follows (the first ones are for the data obtained on increasing temperature changes, and the second ones are for decreasing temperature changes): (○, ●) Δ1; (△, ▲) Δ2; (◭, ◮) Δ2Ala; (▽, ▼) Δ3; (□, ■) Δ4; (◨, ◧) Δ4Ala.

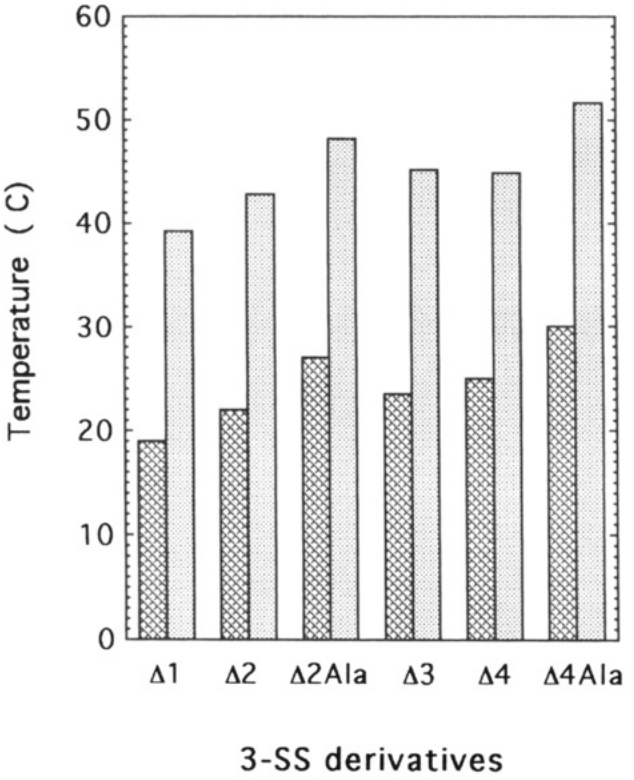

FIGURE 5: Relationship between T_{opt} (cross-hatched) and T_m (dotted) for the 3SS derivatives.

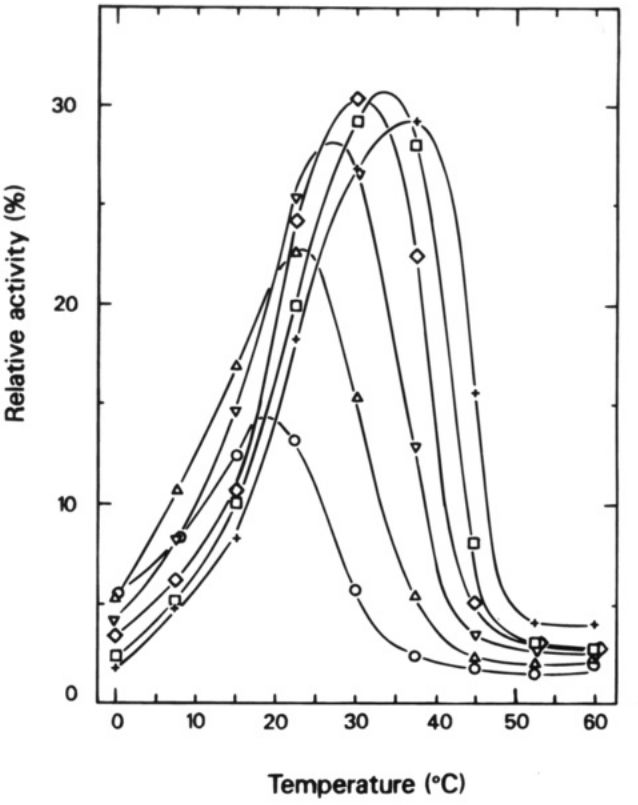

FIGURE 6: Lytic activity of the solution of Δ1 refolded at various temperatures in the absence or presence of glycerol: 0 (○), 10 (△), 20 (▽), 30 (◇), 40 (□), and 50% (+) glycerol (v/v).

temperatures were 19.0, 23.3, 27.0, 30.5, 33.8, and 36.8 °C for the glycerol concentrations of 0, 10, 20, 30, 40, and 50%, respectively. The increment in T_{opt} was 3−4 °C for the increment of 10% in glycerol concentration.

The qualitative features described above can be expressed in a different way if, in Figure 6, we follow the data points for a common temperature value and different glycerol concentrations: at moderately high temperature, say at 37.5 °C, the refolding efficiency was markedly enhanced from nearly zero to 30% by the addition of glycerol while at lower temperature, say at 0 °C, it decreased with the increase in glycerol concentration. Overall, the optimal conditions for the refolding of Δ1 were 30−35 °C in temperature and 30−45% in glycerol concentration.

The HPLC elution profiles of the derivatives reoxidized in the presence of glycerol showed the main peak, whose retention time nearly coincided with that of authentic lysozyme and whose height correlated with the lytic activity of the refolded, crude protein solution (not shown) in a similar way as described before for the case without glycerol.

Then, the refolded and purified Δ1 was subjected to the CD thermal denaturation experiments in the presence of glycerol (Figure 7). Reversible transition curves were obtained in the presence of glycerol, and the transition temperature shifted to the higher side with increasing concentration of glycerol. T_m's were 39.2 (Figure 4), 45.6, and 51.6 ± 0.6 °C for glycerol concentrations of 0, 20, and

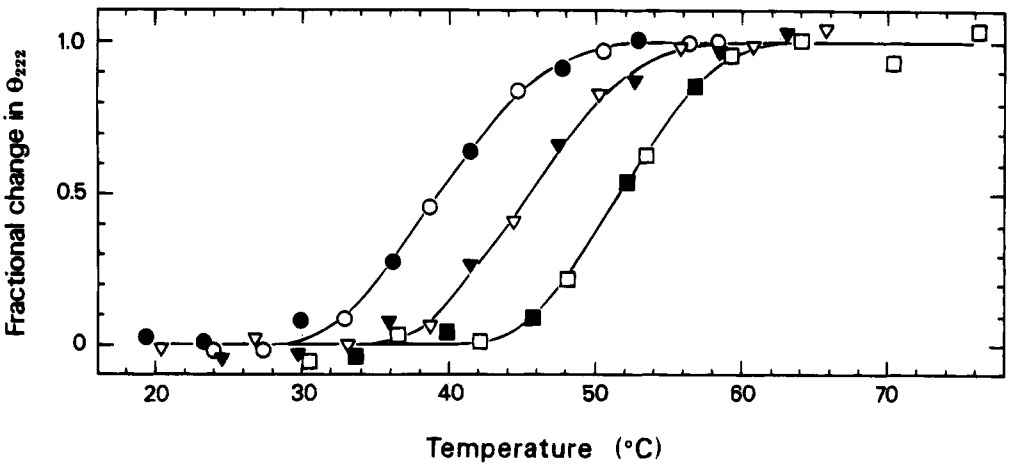

FIGURE 7: Thermal denaturation−renaturation transition curves of Δ1 in the absence (○, ●) or presence of 20% (▽, ▼) or 40% (□, ■) of glycerol. The ordinate shows fractional changes in the mean residue ellipticity at 222 nm. Open and filled symbols indicate the data obtained during increasing and decreasing temperature changes, respectively.

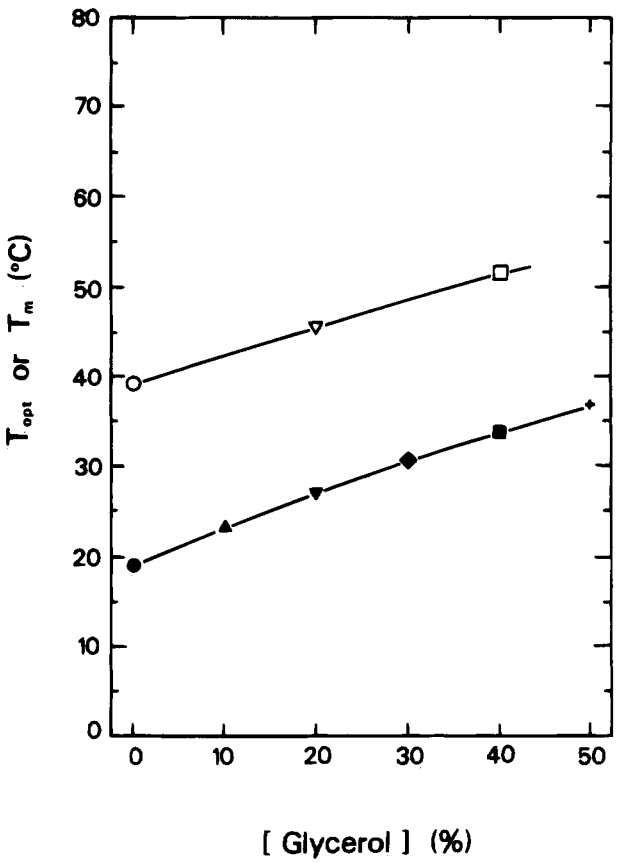

FIGURE 8: Relationship between T_{opt} (lower line) and T_m (upper line) of Δ1 for various concentration of glycerol. The same symbols as in Figures 5 and 6 are used for each glycerol concentration.

40%, respectively. The increment in T_m was 3.2−3.0 °C per 10% increment in glycerol concentration. This value was nearly equal to the increment in T_{opt} described before, and a nearly parallel relationship between T_m and T_{opt} was evident as shown in Figure 8. The differences between T_m and T_{opt} were 20.2, 18.6, and 17.8 °C for glycerol concentrations of 0, 20, and 40%, respectively.

DISCUSSION

Parallelism between T_{opt} and T_m. In this study, it was shown for hen lysozyme 3SS derivatives that there exists a parallel relationship between the optimal temperature for oxidative refolding and the denaturation temperature of the completely refolded state. A set of disulfide bridge-engineered proteins used here was suitable for the demonstration of the relationship with sufficient accuracy: none of the four disulfide bridges is necessary for the refolding of hen lysozyme (Acharya & Taniuchi, 1977; Sawano et al., 1992), and the 3SS derivatives all refolded, although with different efficiencies, into the state that had enough enzymatic activity for the determination of refolding optima as well as the secondary and tertiary structure, which could undergo reversible thermal transition. Such a relationship as demonstrated here will be potentially useful in the optimization of the refolding temperature for expressed, engineered proteins.

The existence of T_{opt} in the "recovered activity"−"refolding temperature" profile and a sharp decrease in the recovered activity with an increase in the refolding temperature are phenomenologically similar to the existence of T_{opt} in the activity−temperature profile of enzymes and probably result from the two factors which have mutually opposing temperature dependence: one is the increase in the rates of elementary reaction steps of oxidative refolding with the increase in temperature, and the other is the destabilization, with the increase in temperature, of the species on the refolding pathway. Here, depending on the stability of the completely refolded state, two cases can be considered: In the first case, the completely refolded species is sufficiently stable, and the reverse reaction through the rate-determining step from the completely refolded species to (an) intermediate species is negligible. In this case, the temperature dependence of the refolding rate will be governed by the stability of the intermediate species. In the second case, the completely oxidized and correctly refolded species is not sufficiently stable, and its reduction, coupled with denaturation, is not negligible. Then, the temperature dependence of the refolding rate as well as the yield will be affected by the stability of the completely oxidized and refolded species.

The oxidative refolding reaction of the hen lysozyme 3SS derivatives studied here was in accord with the second case as described in the Results section. The reaction step leading to the completely refolded Δ1 was shown to be reversible under the present refolding conditions. The rough agreement between the fraction of the recovered activity at 30 °C to

the optimal one (Figure 1) and the fraction of the intact Δ1 remaining after the 50-min incubation at 30 °C (Figure 3) suggests that the major determinant of the temperature dependence of the refolding of Δ1 is the stability of the completely refolded state. The existence of a parallel relationship between T_{opt} and the T_m for the completely refolded states of the 3SS derivatives is therefore considered to be characteristic of the refolded proteins, which have low stability against thermal as well as reduction-coupled denaturation. (Here, we must also note that T_m as well as T_{opt} is generally a function of the concentration of thiol reagents.)

On the other hand, the former case has been clearly demonstrated for the regeneration, i.e., oxidative refolding, of RNase A by Rothwarf and Scheraga (1993a). They showed that RNase A rengerated through multiple pathways and that the rate-determining step involved a rearrangement of 3SS species (Rothwarf & Scheraga, 1993b). They also showed that the regeneration pathways of RNase A depended on the nature of the redox reagent used (Rothwarf & Scheraga, 1993c), and observed correspondingly different temperature dependence of the regeneration rates (Rothwarf & Scheraga, 1993d), which indicated that the temperature dependence is governed by the stability of an intermediate species. It was identified as the 3SS species that was markedly populated at 15 °C. For the oxidative refolding of bovine pancreatic trypsin inhibitor (Creighton, 1977) and that of α-LA (Ewbank & Creighton, 1993a,b), the rate-determining steps have been shown to be an intramolecular two-disulfide rearrangement and that of a two- and three-disulfide rearrangement, respectively.

For authentic (i.e., 4SS) hen lysozyme, it has been shown that the oxidative refolding proceeds through a limited search of intermediates (Ristow & Wetlaufer, 1973; Anderson & Wetlaufer, 1976): two disulfide bonds involving Cys64, Cys76, Cys80, and Cys94 being formed rapidly, followed by the disulfide bond between Cys30 and Cys115, and presumably finally, the disulfide bond between Cys6 and Cys127. This outline of oxidative refolding is nearly the reverse of the reduction pathway for α-LA reported by Ewbank and Creighton (1993a) when the correspondence of the homologous disulfide bridges between the two proteins is taken into consideration, and it may be suggested that the oxidative refolding pathways of the two proteins are roughly similar. However, there also exist differences: In the oxidative refolding of α-LA, multiple one-, two-, and three-disulfide intermediates were generated in contrast to the case for lysozyme; $R^{6/120cam}$, a reduced α-LA in which Cys residues 6 and 120 were blocked with iodoacetamide, did not refold into native (i.e., the remaining three disulfide bridges being in native combinations) state (Ewbank & Creighton, 1993a), whereas in lysozyme, none of the four disulfide bridges is obligatory for the correct folding (Acharya & Taniuchi, 1977; Sawano et al., 1992). At present, unlike in the cases for RNase A, bovine pancreatic trypsin inhibitor, and α-LA due to the lack of the studies for lysozyme that extensively deal in a quantitative way with the kinetic progress of the intermediates in the oxidative refolding pathway, we have little information about either the species involved in a rate-determining step for the oxidative refolding of lysozyme or for that of the refolding of lysozyme 3SS derivatives. It should be noted, however, that the T_m for authentic lysozyme at pH 4 is about 78 °C (Pfeil & Privalov, 1976) while the T_{opt} for its oxidative refolding is 37 °C (Saxena & Wetlaufer, 1970) or 35 °C (this study), resulting in the difference of about 40 °C between T_m and T_{opt}. This value is much larger than the difference of about 20 °C between them for the lysozyme 3SS derivatives studied here. The stability of some intermediate species, most likely 2SS or 3SS, which is much lower than that of the completely refolded authentic lysozyme (=4SS), probably governs the T_{opt}. In other words, the species produced immediately after the rate-determining step will be rapidly stabilized by the formation of the third and/or fourth disulfide bridge(s), making the reduction-coupled denaturation of the completely oxidized and refolded 4SS species almost negligible in the temperature range around, and moderately higher than, T_{opt}.

Stability Differences among the 3SS Derivatives. A simple explanation for the destabilization of protein due to the loss of a disulfide bridge is the increase in the entropy of the denatured state, which is brought about by the change in the organization of covalently closed loops formed by main chain and disulfide bridges. In accordance with this notion, Cooper et al. (1992) have shown that the reduction in T_m resulting from the removal of the 6−127 disulfide bond in hen lysozyme 3SS derivative $CM^{6,127}$ is attributed totally to an increase in the entropy difference between the native and denatured states. In the present case, the entropy increase in the denatured state of each 3SS derivative compared to that of the 4SS form was estimated, based on the equation derived by Lin et al. (1984), to be 17.6, 16.1, 14.5, and 14.8 cal/K·mol for Δ1−Δ4, respectively. If there is no change between 3SS and 4SS forms in the enthalpy as well as the entropy difference for the unfolding reaction except for this entropy increase, the amount of the decrease in T_m with the removal of a single disulfide bridge is predicted to be Δ1 > Δ2 > Δ4 ≳ Δ3. The present result of CD T_m measurement agreed with this prediction. However, the change between the 3SS and the 4SS forms in the enthalpy difference as well as in the entropy difference for the denaturation reaction was observed in our preliminary differential scanning calorimetry experiment (Tachibana, Oka, Fukada, Takahashi, Sorai, unpublished results). Also, Kuroki et al. (1992) have reported that the destabilization of the disulfide-engineered human lysozyme, which corresponds to Δ4 studied here, is not explained by the change in entropy difference but rather by the change in enthalpy difference. Complete interpretation of the stability change among the 3SS derivatives in terms of the structural features of the lysozyme molecule awaits the result of a detailed thermodynamic investigation.

The Cys→Ala 3SS derivatives (Δ2Ala, Δ4Ala) had higher T_m than their counterpart Cys→Ser derivatives (Δ2, Δ4). The environment of the residue positions in authentic lysozyme where Cys residues are replaced in these 3SS derivatives (30, 115, 76, 94) is inside the molecule (Shrake & Rupley, 1973). Therefore, the stabilization of the Ala derivatives relative to the Ser derivatives can be qualitatively explained by the relationship between the stability of the engineered protein and the hydrophobicity of substituted residues (Yutani et al., 1987). The difference in T_m observed in this study, about 6 °C, however is much smaller than that (27 °C) reported for a single disulfide bond derivative of the bovine pancreatic trypsin inhibitor with the remaining Cys residues replaced by either Ser or Ala (Darby et al., 1991; van Mierlo et al., 1991; Staley & Kim, 1992; the value was corrected for the effect of the substitution or the addition of residues

other than Cys→Ser/Ala replacement according to the description in Staley and Kim). We must note that, in the case of human lysozyme 3SS derivatives, a Cys77,95→Ser mutant has been reported to be more stable by 2.3 °C in T_m than a Cys77,95→Ala mutant, probably due to a hydrogen bond between the two Ser residues (Yamada et al., 1994).

Refolding Efficiency and the Effect of Glycerol. The maximal refolding efficiency shown in Figure 1, evaluated by apparent lytic activity of the reoxidized (i.e., not purified) protein solution, was significantly higher for Δ4, Δ2Ala, and Δ4Ala than for Δ1, Δ2, and Δ3. The same situation is observed for the refolding efficiency at almost any refolding temperature. The reason of the high apparent activity for Δ4 is partly explained by the high activity of purified Δ4 (Sawano et al., 1992). Although the activities of the purified Δ1, Δ2, and Δ3 were 60−90% of the authentic lysozyme, the activity of the purified Δ4 was 130−140% of authentic lysozyme. [This high activity may be related with the proximity of the disulfide bridge Cys76−Cys94, which is removed in Δ4, to Trp62. The replacement of Trp62 with Tyr, Phe, or His has been shown to exhibit enhanced bacteriolytic activity (Kumagai & Miura, 1989), and it was suggested that the smaller size of the aromatic ring at the 62nd position may favor the lytic process.] In the case of Δ2Ala, the activity of the purified protein (about 72% of authentic lysozyme) was similar to that of Δ2 (60−76%). Therefore, the apparently higher activity of the reoxidized solution of Δ2Ala must be related with an increased number of correctly refolded protein molecules compared to Δ2. Likewise, the similar level of refolding efficiency between Δ4 and Δ4Ala combined with the lower activity of the purified Δ4Ala compared to that of the purified Δ4 indicates an increased number of correctly refolded Δ4Ala compared to Δ4. Altogether, the Cys→Ala derivatives studied in this study had a higher refolding efficiency as well as a higher stability than their counterpart Cys→Ser derivatives.

The appearance of significant refolding yield at a progressively higher temperature side with increasing glycerol concentration in the reoxidation reaction of Δ1 (Figure 5) can be explained by the stabilization, during reoxidation reaction, of a completely refolded state with glycerol. The observed amount of stabilization by glycerol of the refolded and purified Δ1 (3.2−3.0 °C per 10% increment in glycerol concentration) as well as the increase in T_{opt} with the increase in glycerol concentration (4−3 °C per 10% increment in glycerol concentration) were significantly higher than that reported for chymotrypsinogen (1.1−0.5 °C), RNase (2.4−1.7 °C) (Gekko & Timasheff, 1981b), and authentic lysozyme (0.7−1.5 °C) (Gekko, 1982). We also carried out the control refolding experiment for authentic lysozyme in the presence of glycerol (not shown). The increase in T_{opt} was about 2 °C per 10% increment in glycerol concentration and was significantly lower than that for Δ1. The enhanced amount of stabilization for Δ1 can be explained as follows: It has been shown that the folded state of protein is stabilized by glycerol with the mechanism of preferential hydration (Gekko & Timasheff, 1981a). In the mechanism, the unfavorable interaction between glycerol and protein tends to minimize the surface of contact between them, thereby stabilizing the compact, folded state relative to the extended, unfolded state. In the present case, the compactness of the folded state of Δ1 is considered to be nearly the same as that of authentic lysozyme since the amount of secondary structures, that of enzymatic activity, and the elution position on RPHPLC were nearly the same with those of authentic lysozyme. On the other hand, the unfolded state of Δ1, which has three disulfide bridges, is naturally expected to be less compact compared to that of authentic lysozyme, which has four disulfide bridges. Therefore, the amount of free energy increase for the unfolded state due to the unfavorable interaction with glycerol is expected to be higher for Δ1 than for authentic lysozyme, resulting in an increased amount of stabilization of the folded state of Δ1 compared to that of authentic lysozyme.

ACKNOWLEDGMENT

We thank Profs. K. Nakamae and Y. Nishizuka for encouragement and support, Prof. K. Akasaka for discussions, critical reading of the manuscript, and the facilities for CD measurements, and Mr. T. Oka for the preparation of Δ4.

REFERENCES

Acharya, A. S., & Taniuchi, H. (1976) *J. Biol. Chem. 251*, 6934−6946.
Acharya, A. S., & Taniuchi, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2362−2366.
Acharya, A. S., & Taniuchi, H. (1982) *Mol. Cell. Biochem. 44*, 129−148.
Anderson, W. L., & Wetlaufer, D. B. (1976) *J. Biol. Chem. 251*, 3147−3153.
Cooper, A., Eyles, S. J., Radford, S. E., & Dobson, C. M. (1992) *J. Mol. Biol. 225*, 939−943.
Creighton, T. E. (1977) *J. Mol. Biol. 113*, 275−293.
Darby, N. J., van Mierlo, C. P. M., & Creighton, T. E. (1991) *FEBS Lett. 279*, 61−64.
Ewbank, J. J., & Creighton, T. E. (1993a) *Biochemistry 32*, 3677−3693.
Ewbank, J. J., & Creighton, T. E. (1993b) *Biochemistry 32*, 3694−3707.
Gekko, K. (1982) *J. Biochem. 91*, 1197−1204.
Gekko, K., & Timasheff, S. N. (1981a) *Biochemistry 20*, 4677−4686.
Gekko, K., & Timasheff, S. N. (1981b) *Biochemistry 20*, 4667−4676.
Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319−326.
Heinrikson, R. L., & Meredith, S. C. (1984) *Anal. Biochem. 136*, 65−74.
Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 199−203.
Kumagai, I., & Miura, K.-I. (1989) *J. Biochem. 105*, 946−948.
Kuroki, R., Inaka, K., Taniyama, Y., Kidokoro, S., Matsushima, M., Kikuchi, M., & Yutani, K. (1992) *Biochemistry 31*, 8323−8328.
Lin, S. H., Konishi, Y., Denton, M. E., & Scheraga, H. A. (1984) *Biochemistry 23*, 5504−5512.
Miki, T., Yasukochi, T., Nagatani, H., Furuno, M., Orita, T., Yamada, H., Imoto, T., & Horiuchi, T. (1987) *Protein Eng. 1*, 327−332.
Mitraki, A., & King, J. (1989) *Bio/Technology 7*, 690−697.
Nagai, K., & Thøgersen, C. (1987) *Methods Enzymol. 153*, 461−481.
Pfeil, W., & Privalov, P. L. (1976) *Biophys. Chem. 4*, 23−32.
Radford, S. E., Woolfson, D. N., Martin, S. R., Lowe, G., & Dobson, C. M. (1991) *Biochem. J. 273*, 211−217.
Ristow, S. S., & Wetlaufer, D. B. (1973) *Biochem. Biophys. Res. Commun. 50*, 544−550.
Rothwarf, D. M., & Scheraga, H. A. (1993a) *Biochemistry 32*, 2671−2679.

Rothwarf, D. M., & Scheraga, H. A. (1993b) *Biochemistry 32*, 2680−2789.

Rothwarf, D. M., & Scheraga, H. A. (1993c) *Biochemistry 32*, 2690−2697.

Rothwarf, D. M., & Scheraga, H. A. (1993d) *Biochemistry 32*, 2698−2703.

Sawano, H., Koumoto, Y., Ohta, K., Sasaki, Y., Segawa, S., & Tachibana, H. (1992) *FEBS Lett. 303*, 11−14.

Saxena, V. P., & Wetlaufer, D. B. (1970) *Biochemistry 9*, 5015−5023.

Shrake, A., & Rupley, J. A. (1973) *J. Mol. Biol. 79*, 351−371.

Staley, J. P., & Kim, P. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1519−1523.

Tachibana, H., Sasaki, Y., Sawano, H. & Kitakawa, M. (1990) *Protein Eng. 3*, 371.

Taniyama, Y., Yamamoto, Y., Nakao, M., Kikuchi, M., & Ikehara, M. (1988) *Biochem. Biophys. Res. Commun. 152*, 962−967.

van Mierlo, C. P. M., Darby, N. J., Neuhaus, D., & Creighton, T. E. (1991) *J. Mol. Biol. 222*, 373−390.

Yamada, H., Kanaya, E., Ueno, Y., Ikehara, M., Nakamura, H., & Kikuchi, M. (1994) *Biol. Pharm. Bull. 17*, 612−616.

Yutani, K., Ogasahara, K., Tsujita, T., & Sugino, Y. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4441−4444.