Colloid Polym Sci 278:979–985 (2000)

SHORT COMMUNICATION

Y. Moriyama
K. Hirao
K. Takeda

Conformational changes of lysozymes with different numbers of disulfide bridges in sodium dodecyl sulfate solutions

Received: 17 September 2000
Accepted: 24 March 2000

Y. Moriyama · K. Hirao · K. Takeda (✉)
Department of Applied Chemistry
Okayama University of Science
1-1 Ridai-cho Okayama 700-0005
Japan
e-mail: takeda@dac.ous.ac.jp
Tel.: +81-086-2569553
Fax: +81-086-2569553

Abstract Four disulfide bridges of hen egg-white lysozyme were selectively reduced to obtain its derivatives with three, two, and zero disulfide bridges (designated as 3SS, 2SS, and 0SS lysozymes, respectively). The 3SS lysozyme maintained the native conformation at pH 7.0 and 3.0. Even upon the reduction of two disulfide bridges, the protein conformation still remained unchanged at pH 7.0. Upon the reduction of all four disulfide bridges, the helicity, $[\theta]_{270}$, and tryptophan fluorescence changed at pH 3.0 as well as at pH 7.0. The helicity of each derivative increased in a solution of sodium dodecyl sulfate (SDS). The SDS-induced helicity of the 0SS lysozyme was lower at pH 7.0 and higher at pH 3.0 than that of the intact lysozyme with four disulfide bridges. The helix formation appears to occur in originally nonhelical parts in each derivative at pH 7.0. In the cases of the 2SS and 0SS lysozymes at pH 3.0, however, some of the helices appear to be reformed also at moieties where the original helices are disrupted upon the cleavage of disulfide bridges.

Key words Lysozyme · Sodium dodecyl sulfate · Selective disulfide bond cleavage · Secondary structure · Fluorescence

Introduction

Many studies have been conducted on the interaction between proteins and surfactants [1–3]. It has become clear that several proteins adopt conformations with higher helix contents than their native states upon the addition of ionic surfactants such as sodium dodecyl sulfate (SDS) [4, 5]. This is in marked contrast with denaturations by other denaturants. Hen egg-white lysozyme is one of the proteins which can be converted in part to the helical conformation in SDS solution [6, 7].

Hen egg-white lysozyme has been a target protein to investigate protein structure. Since the structure of lysozyme was solved by X-ray diffraction [8–10], various studies have been made on the protein. Mckenzie and White have recently reviewed the lysozyme conformation as follows [11]. The protein has a deep cleft on one side. The cleft divides the protein molecules roughly into two lobes. One lobe (residues 1–39 and 85–129) contains four helices (helix A, residues 4–15; helix B, 24–36; helix C, 88–99; helix D, 108–115) and one single-turn 3_{10} helix (119–124). The other lobe (residues 40–84) contains a three-stranded antiparallel β-pleated sheet (residues 42–60), a small β sheet (between 1–2 and 39–40), and a single-turn 3_{10} helix (79–84). These secondary structures of the protein must be maintained more or less by the existence of four disulfide bridges.

Quite a few investigations have been made so far focusing on the contribution of the bridges to the lysozyme conformation; however, there are contradictions in the previous reports. It has been reported that lysozyme maintains a native conformation upon the loss of a single disulfide bridge between Cys6 and Cys127

[12–14] and that fully reduced lysozyme is greatly unfolded relative to the native protein [15, 16]. In contrast, it has also been reported that not only partially reduced lysozyme [17] but also fully reduced lysozyme [18–20] assumes conformational states that resemble the native conformation.

In the present article, a comparative study of the conformation was made among four types of egg-white lysozymes with four (intact), three, two, and zero disulfide bridges, (designated as intact, 3SS, 2SS, and 0SS lysozymes, respectively) under consistent conditions. The formation of a helical structure was drastically induced in each lysozyme derivative in SDS solution, although the profile of the formation in the 0SS lysozyme was different from those in the others.

Experimental

Crystalline hen egg-white lysozyme was obtained from Miles Laboratories. SDS and 1-anilinonaphthalene-8-sulfonic acid (ANS) were purchased from BDH and Sigma, respectively. A phosphate buffer (pH 7.0) and a glycine–HCl buffer (pH 3.0) of ionic strength 0.014 were used in the SDS denaturation to keep the critical micelle concentration in ratter high concentrations [21]. On the other hand, in the guanidine denaturation experiment, a 0.1 M phosphate buffer was used [22].

Stepwise reduction methods of the four disulfide bridges in lysozyme have been studied by many investigators [17, 18, 20, 23–29]. In the present study, only the Cys6-Cys127 disulfide bridge was selectively reduced, according to the method of Acharya and Taniuchi [17]. Two of the four bridges were reduced according to the method by Azari [29]. It has been suggested that Cys76-Cys94 opens following Cys6-Cys127 under a condition to reduce two bridges of lysozyme [17]. All four bridges were reduced according to the method of Crestfield et al. [23]. Although any blocking of the resulting free sulfhydryl groups might cause another effect on the protein conformation, the free sulfhydryl groups were carboxyamidomethylated in each case in the usual manner to prevent a relinkage; thereafter, each derivative was lyophilized. Each derivative showed a single peak in the reverse-phase chromatography by use of TSKgel Phenyl-5PW RP column (Tosoh). As a result, each derivative was confirmed to be pure up to the same level as the disulfide-intact lysozyme.

Circular dichroism (CD) and fluorescence measurements were made at 25 °C with a Jasco J-600 spectropolarimeter [30] and a Jasco FP-777 spectrofluorometer, respectively. Protein concentrations were determined using an extinction coefficient at 280 nm of 38,000 M^{-1} cm^{-1} for the intact, 3SS, and 2SS lysozymes and 34,000 M^{-1} cm^{-1} for the 0SS lysozyme [19, 31]. Throughout the measurements, the concentration of each lysozyme was kept at 10 μM, except for 50 μM in the near-UV CD measurement. The helicity of lysozyme has often been estimated [6, 7, 20, 31] by curve-fitting of the CD spectrum using the reference spectra as determined by Chen et al. [32]. The helicities of the present lysozyme derivatives were also estimated by the same method. In the measurements of the trypophan and ANS fluorescence spectra, the excitation wavelengths were 300 and 360 nm, respectively. The excitation and emission spectra were corrected using the rhodamine B quantum counter (Jasco QTC-111) attached to the apparatus. The ANS concentration was determined using an extinction coefficient of 5000 M^{-1} cm^{-1} at 350 nm [33].

Results and discussion

The effect of the selective cleavages of the disulfide bridges on the lysozyme conformation

The purpose of the present work is to examine the relationship between the lysozyme conformation and the remaining disulfide bridges in a surfactant solution. Prior to the examination of this relationship, the conformational change of lysozyme with the stepwise reduction of the four disulfide bridges was analyzed. As mentioned in the Introduction, different results have been reported so far on the effect of the extent of reduction of the disulfide bridges on the lysozyme conformation. Keeping these different previous reports in mind, the present measurements were carried out.

CD spectra of the intact lysozyme, the 3SS lysozyme the 2SS lysozyme, and the 0SS lysozyme in far-UV and near-UV regions are shown in Fig. 1. The spectra of the 3SS and 2SS lysozymes resembled those of the intact lysozyme in both the far-UV and the near-UV regions at pH 7.0. Only the spectra of the 0SS lysozyme differed from those of the intact lysozyme in both the far-UV and the near-UV regions. The present results indicate that not only the secondary structure but also the tertiary structure of lysozyme is maintained upon the loss of two disulfide bridges at Cys6-Cys127 and probably Cys76-Cys94 and that only the fully reduced lysozyme is unfolded relative to the native state. The helicity of each derivative was estimated by a usual curve-fitting of the CD spectrum in the far-UV region. The helicity of the intact lysozyme agrees approximately with those which have previously been estimated by the same method [6, 7]. The helicity of the 0SS lysozyme is similar to that estimated by the same method [31]. It was found in the present study that the helicities of the 3SS and 2SS lysozymes were identical with that of the intact lysozyme. Only the helicity of the 0SS lysozyme was lower than those of the others. The cleavage of the two disulfide bridges at Cys6-Cys127 and Cys76-Cys94 does not induce a disruption of the helical structures in lysozyme. The disruption of the helical structures is caused by further cleavages of the disulfide bridges at Cys30-Cys115 and Cys64-Cys80. In other words, the helical structures of lysozyme are tightly maintained by these two disulfide bridges. These results are summarized as a function of the number of remaining disulfide bridges in Fig. 2. This figure also includes changes in $[\theta]_{270}$, the maximum wavelength of the tryptophan fluorescence emission spectrum (λ_{max}), and the fluorescence intensity of the hydrophobic fluorescent dye, ANS, around 480 nm for each lysozyme derivative. The λ_{max} position was observed around 337 nm for the intact lysozyme and the 3SS lysozyme, while it was at 339 and 341 nm for the 2SS and 0SS lysozymes, respectively.

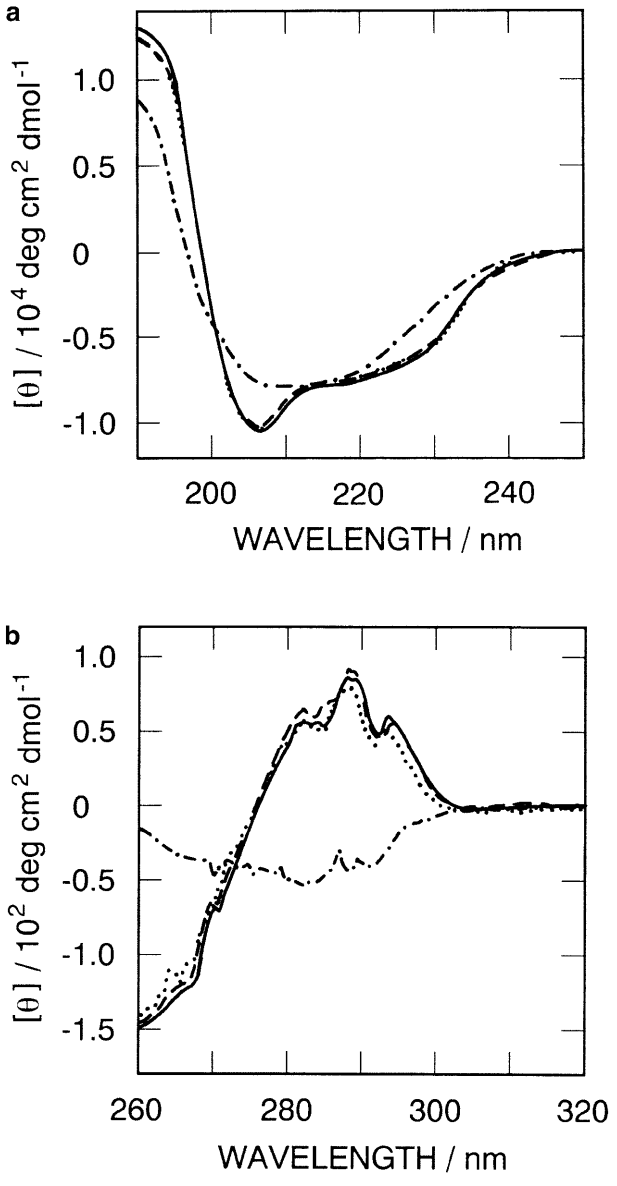

Fig. 1 Circular dichroism spectra of intact (——), three-disulfide-bridged (3SS) (— —), two-disulfide-bridged (2SS) (···), and zero-disulfide-bridged (0SS) (–·–) lysozymes in **a** the far-UV and **b** the near-UV regions at pH 7.0

The values of the helicity and $[\theta]_{270}$ remained unchanged until the cleavage of two disulfide bridges. On the other hand, the intact lysozyme was found to have a binding site for the hydrophobic dye, ANS. Contrary to our expectation, the fluorescence of ANS became maximal upon the reduction of one disulfide bridge, Cys6-Cys127. Upon the reduction of all four disulfide bridges, on the other hand, various changes were induced. The helicity decreased and the value of $[\theta]_{270}$ changed greatly. The λ_{max} position also shifted, reflecting a large-scale change of the conformation.

It is well known that hen egg-white lysozyme is homologous with α-lactalbumin in their amino acid sequences and conformations [34, 35]. Noting the homology between the two proteins, unfolding or refolding transitions of the two intact proteins have frequently been compared [12, 35–37]. In the case of α-lactalbumin, various conformational changes drastically occur depending on the remaining number of disulfide bridges [24–28, 38–40]. The present study also indicates some differences between the two proteins. The result of λ_{max} in Fig. 2 shows that Trp123 in the vicinity of the disulfide bridge at Cys6-Cys127 in lysozyme appears not to be exposed to solvent upon the reduction of two bridges, although Trp118 of α-lactalbumin (corresponding to Trp123 in lysozyme) is exposed upon the reduction of only Cys6-Cys120 [40]. The hydrophobic dye, ANS, binds even to the intact lysozyme, although the dye hardly binds to the intact α-lactalbumin [40]. In the lysozyme molecule, a hydrophobic box, which can bind a substrate [9, 10], is formed by Tyr20, Tyr23, Trp28, Trp108, Trp111, Leu17, Ile98, and Met105. It has been reported that the enzymatic activity of lysozyme appreciably decreases upon the reduction of Cys6-Cys127 [9, 10]; therefore, it appears likely that a part of the hydrophobic box in the 3SS lysozyme is adequately exposed to a solvent in order to bind the hydrophobic dye. However, in the case of the 0SS lysozyme, not only a part of the box but also other hydrophobic regions might be exposed. Then this derivative might be precipitated probably by sandwiching the dye.

In the case of α-lactalbumin, various changes occur upon the reduction of two disulfide bridges, Cys28-Cys111 in addition to Cys6-Cys120, and then the helicity of this protein apparently decreases [40]. In contrast to this, the helicity, $[\theta]_{270}$, and λ_{max} of lysozyme hardly change upon the reduction of two disulfide bridges. These results seem to indicate that the conformation of lysozyme itself is rigidly formed compared with that of α-lactalbumin. On the other hand, the Cys76-Cys94 bridge (corresponding to the Cys73-Cys91 bridge in α-lactalbumin) has been suggested to be secondly reduced in lysozyme [17], although the Cys28-Cys111 bridge (corresponding to Cys30-Cys115 bridge in lysozyme) has been considered to be secondly reduced in α-lactalbumin [27, 28]. Then, there is the possibility that the 2SS lysozyme essentially differs from the 2SS α-lactalbumin in the location of the two remaining bridges.

The effect of the selective cleavages of the disulfide bridges on the conformational changes in SDS solution

The conformations of lysozymes with zero to four disulphide bridges were examined in a solution of an

anionic surfactant, SDS. The SDS concentration dependences of the λ_{max} shift of the tryptophan fluorescence for the four derivatives are shown in Fig. 3. The λ_{max} positions of the intact and the 3SS lysozymes did not change in SDS solution. This protein has six tryptophan residues, which are scattered all over the molecule. In the surfactant solution, the conformations of the intact and 3SS lysozymes remained unchanged and the tertiary structures seem to be preserved. In other words, the existence of four or three disulfide bridges protects the lysozyme from disruption of the tertiary structure by SDS. On the other hand, the λ_{max} positions of the 2SS and 0SS lysozymes, which were shifted to higher wavelengths by the cleavage of the disulfide bridges, returned approximately to that of the intact lysozyme in SDS solution.

The helicity changes of the four derivatives in SDS solution are shown in Fig. 4. The helicity of each derivative increased in SDS solution. The guanidine concentration dependence of the helicity of each derivative is shown in Fig. 5. The helicities of the 2SS and 0SS lysozymes began to decrease at lower guanidine concentrations than those of the others. There is little difference between the intact and 3SS lysozymes in the guanidine denaturation profiles, although the 3SS lysozyme has been reported to lose stability in the thermal denaturation at pH 3.8 [23, 41]. In the present study, a clear difference between the 3SS and 2SS lysozymes was observed in the secondary structure stabilities in the guanidine denaturation and in the λ_{max} shifts in SDS solution (Fig. 3); however, such a difference was not

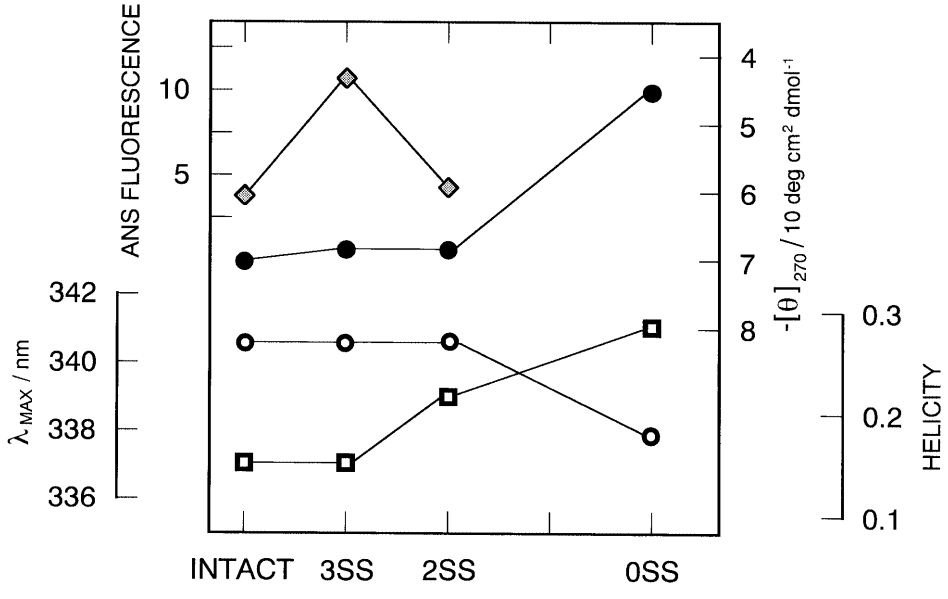

Fig. 2 Changes in helicity (○), $[\theta]_{270}$ (●), λ_{max} (□), and 1-anilinonaphthalene-8-sulfonic acid (*ANS*) fluorescence (◈) of lysozyme with an increase in the number of reduced disulfide bridges at pH 7.0

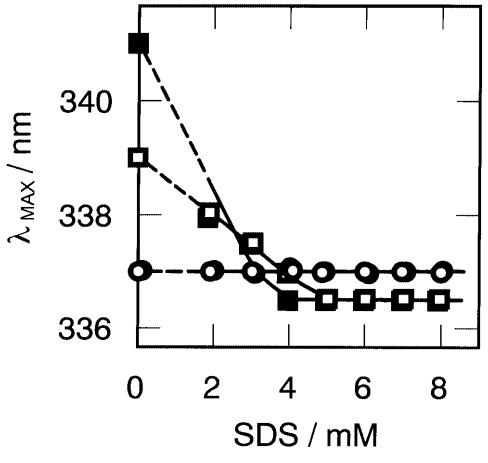

Fig. 3 Sodium dodecyl sulfate (*SDS*) concentration dependence of λ_{max} of intact (○), 3SS (●), 2SS (□), and 0SS (■) lysozymes at pH 7.0

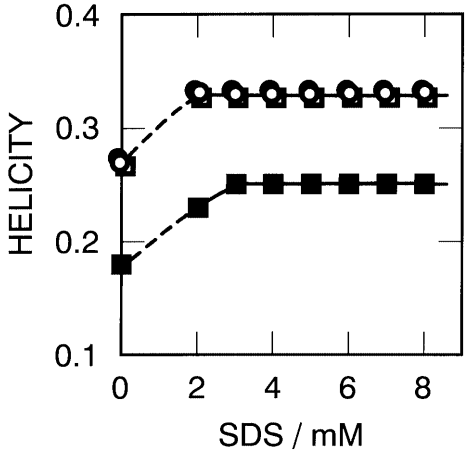

Fig. 4 SDS concentration dependence of the helicities of intact (○), 3SS (●), 2SS (□), and 0SS (■) lysozymes at pH 7.0

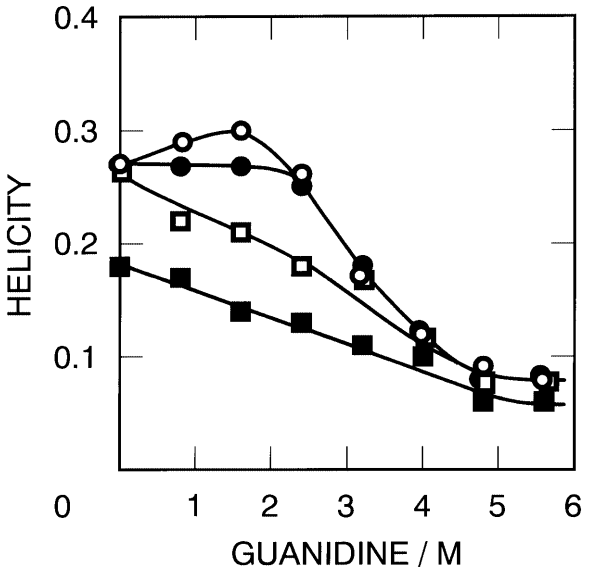

Fig. 5 Guanidine concentration dependence of the helicities of intact (○), 3SS (●), 2SS (□), and 0SS (■) lysozymes at pH 7.0

observed in the helicity changes of the two derivatives in SDS solution.

The helicity of the intact lysozyme increased in SDS solution, as stated previously. This indicates that originally nonhelical parts are converted to the helical structure. The helicities of the 3SS and 2SS lysozymes increased similarly to that of the intact lysozyme in the surfactant solution. The helical structures must be formed in the same nonhelical parts. On the other hand, the helicity of the 0SS lysozyme also increased in SDS solution; however, the final helicity of the 0SS lysozyme was smaller than those of the others in the surfactant solution. The increment of the helicity in the 0SS lysozyme approximately equals those in the other lysozymes. This fact indicates that the helical structures are formed in the same nonhelical parts in the 0SS lysozyme as in the intact lysozyme and that the helical structures are not formed in moieties where the original helical structures are disrupted by the cleavage of the disulfide bridges. In the light of the result in Fig. 3, the helix formation accompanies a large-scale tertiary structural change in the cases of the 2SS and 0SS lysozymes.

The N-terminal helical segment of the reduced α-lactalbumins, Lys5-Glu11, has been proposed to be disrupted in SDS solution [40]. Against this, the helical segment, Arg5-His15, of the reduced lysozymes appears not to be disrupted in the same surfactant solution, since the final helicities of the 3SS and 2SS lysozymes are identical with that of the intact lysozyme. One of the reasons for this must be a difference between sequences in the middle moieties of the corresponding segments in both proteins: a sequence -Ala9-Ala10-Ala11- in lysozyme against -Phe9-Arg10-Glu11- in α-lactalbumin. The middle moiety of the lysozyme segment cannot interact electrostatically with dodecyl sulfate ions. In addition, the stability difference of the helical segment in the surfactant solution might also arise from the fact that Arg5-His15 in lysozyme is longer than Lys5-Glu11 in α-lactalbumin.

These statements refer to results, obtained at pH 7.0. The same examination was also made at pH 3.0. The SDS concentration dependences of the λ_{max} shift for the four lysozymes is shown in Fig. 6. The turbidity range of the SDS concentration spreads out and then the measurements were made above 3 mM SDS at this pH. The λ_{max} positions of the intact and 3SS lysozymes were not affected by the pH change; however, the λ_{max} positions of the 2SS and 0SS derivatives shifted to higher wavelengths upon the cleavage of the disulfide bridges at pH 3.0 compared with those at pH 7.0 (see the data at 0 mM SDS in Figs. 3, 6). In the SDS solution of pH 3.0, on the other hand, the λ_{max} positions of the intact and 3SS derivatives hardly shifted, but those of the 2SS and 0SS lysozymes shifted greatly to lower wavelengths (Fig. 6). It can be expected that the SDS binding causes larger tertiary structural changes of the 2SS and 0SS lysozymes at pH 3.0 than at pH 7.0.

At pH 3.0, the far-UV and near-UV CD spectra of the intact lysozyme approximately agreed with those of it at pH 7.0, although the near-UV spectrum showed a little red shift at the acidic pH (not shown). Also at pH 3.0, an appreciable difference was not observed between the spectra of the intact and 3SS lysozymes in both regions, while only the far-UV CD spectrum of the 0SS lysozyme apparently differed from that of the intact lysozyme. At this pH, the near-UV CD strength of the 2SS lysozyme weakened as a whole compared with that of the intact lysozyme and then the spectrum of the 0SS

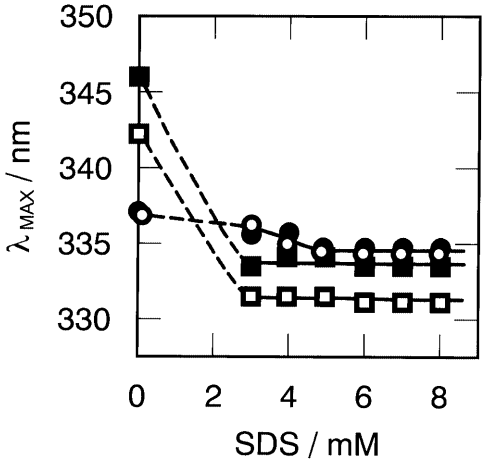

Fig. 6 SDS concentration dependence of λ_{max} of intact (○), 3SS (●), 2SS (□), and 0SS (■) lysozymes at pH 3.0

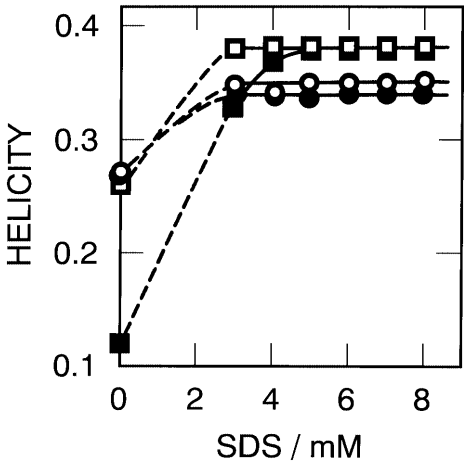

Fig. 7 SDS concentration dependence of the helicities of intact (○), 3SS (●), 2SS (□), and 0SS (■) lysozymes at pH 3.0

lysozyme furthermore faded. The λ_{max} and the near-UV CD data indicate that the 3SS derivative maintains a tertiary structure similar to that of the intact protein and that the tertiary structure appreciably changes upon the reduction of more than two disulfide bridges also at pH 3.0. These observations for the 3SS lysozyme agree with those obtained by other investigators [12–14], who have concluded that the loss of a single disulfide bridge at Cys6-Cys127 has no major effect on the conformation of the protein. Interestingly, the present results suggest that the 2SS lysozyme at pH 3.0 adopts a molten globule state with a nativelike secondary structure but a distorted tertiary structure [27, 28, 35, 39, 40, 42, 43].

The helicity changes of the four derivatives in SDS solution of pH 3.0 are shown in Fig. 7. The helicity of each derivative increased in SDS solution. The molten globule state of the 2SS lysozyme was disrupted. The increasing profiles of the helicities of the intact and 3SS lysozymes were identical with those at pH 7.0; however, the change in pH causes a great difference between the increasing profiles of the helicities of the 2SS and 0SS lysozymes. The helicity of the 2SS lysozyme, which was not so different from that of the intact lysozyme at 0 mM SDS, increased more than that of the intact lysozyme in SDS solution. Although the cleavage of the four disulfide bridges caused a greater loss of helicity at pH 3.0 than at pH 7.0, the helicity of the 0SS lysozyme increased to the same extent as that of the 2SS lysozyme at pH 3.0. The helix formation appears to occur in originally nonhelical parts in the intact and 3SS lysozymes in SDS solution at pH 3.0 as at pH 7.0. In the cases of the 2SS and 0SS lysozymes at pH 3.0, however, some of helices might also be formed at moieties where the original helices are disrupted by the cleavage of the disulfide bridges. The same recovery of the helical structure, lost by the cleavage of the disulfide bridges, has been observed in bovine serum albumin [22, 44, 45], which has most frequently been adopted to study the interaction with surfactants [1–3, 46]; however, such a recovery does not occur in lysozyme at pH 7.0. At neutral pH, the conformation of the 0SS lysozyme seems to be considerably stabilized by newly arranged interactions, some of which do not appear at acidic pH. In addition, the interaction with the anionic surfactant, SDS, favors a decrease in the number of negatively charged residues at acidic pH. Such a favorable situation appears to make it possible to form more helical structures at pH 3.0 than at pH 7.0.

The present comparison of SDS effects at pH 7.0 and 3.0 clearly indicates that the conformation of lysozyme itself is rigidly maintained even upon the loss of all four disulfide bridges, especially at neutral pH. Furthermore, the present study shows that the conformation of fully reduced lysozyme is clearly different from that of the intact protein at pH 7.0 and 3.0. In SDS solution, the fully reduced lysozyme behaves quite differently from the intact protein at neutral pH as well as at acidic pH. The helicity increment of the 0SS lysozyme in the surfactant solution is larger at pH 3.0 than at pH 7.0, probably because the relative charge of the protein becomes more positive at acidic pH, creating more favorable conditions for interaction with dodecyl sulfate ions.

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