1P125**TFE-induced structures in lysozyme variants lacking some of the disulfide bonds determined by the residue-specific H-D exchange method.**○Naoki Tokunaga¹, Yasuo Noda¹, Hideki Tachibana², Shin-ichi Segawa¹¹Sch. Sci. & Tech., Kwansei Gakuin Univ., ²Fac. Sci., Kobe Univ

Lysozyme variants lacking some of the disulfide bonds were studied in solutions containing TFE (trifluoroethanol) which is known to induce helical structure. The helical content induced by the addition of 50% TFE varied with the number of remaining S-S bonds, that is to say, it was largest for OSS-variant, while it was intermediate for 2SS-variants and minimized for wild-type. This indicates that remaining S-S bonds suppress the formation of excess helical structures, but instead conformational restrictions are likely to induce some collapsed structures in other parts. In order to see regions where hydrogen bonded structures are induced, protection factors against H-D exchange reactions of amide protons were measured by NMR at a residue level. The protection factors significantly increased in the regions of residues L8-K13, L25-E35, Y53-I58 and I88-K96 in both variants, but the values of protection factors themselves were larger in 2SS-variant than in OSS-variant. This means that some regions protected in the α -domain (L8-K13, L25-E35) are stabilized more by the presence of a cluster of residues within the β -domain in 2SS[64-80, 76-94], in spite of the absence of tertiary structure. Further it seems interesting that the interface region between α - and β -domains is protected in both variants. Another 2SS-variant, 2SS[6-127,64-80] is interesting to be investigated, because its tertiary structure is induced by the addition of 30% glycerol. TFE-induced ordered structure was studied for the comparison with the results mentioned above.

1P127**Observation of structure formation in the oxidative refolding of ribonuclease A**

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In the oxidative refolding pathways of proteins that have several SS linkages, SS formation and SS reshuffling reactions progress simultaneously. When sulfur reagents, such as glutathione and DTTred, are used in the oxidative refolding experiments of proteins, the structure formation coupled with the SS reshuffling is difficult to be observed because the SS formation reaction proceeds slowly by use of the sulfur reagents. However, we found that the use of a water-soluble selenium reagent, called DHSox, allows separation of the SS formation and SS reshuffling reactions because the SS formation reaction proceeds faster than the SS reshuffling reaction. To observe the coupling of the structure formation and the SS reshuffling, we studied the oxidative refolding pathways of ribonuclease A by using DHSox as an oxidizing reagent. The SS formation reaction (oxidation) ended up in 1 minute after addition of three equivalents of DHSox to the solution containing the reduced form of ribonuclease A (R) at 25 degree and pH 8.0, and the SS reshuffling reaction proceeded subsequently. HPLC analysis revealed that after one minute the reaction solution contained R, 1S, 2S, 3S, and 4S intermediates but they gradually transformed to two folding intermediates (des[40-95] and des[65-72] intermediates having three native SS bonds) and N (the native state) in 5h. According to UV spectral analysis, the two 3S intermediates were shown to have a similar structure to N. Structure formation during the slow SS reshuffling reaction will be discussed.

1P126**Effects of glycerol on kinetics of the disulfide bonds regeneration of 2SS-variant of lysozyme, 2SS[6-127, 64-80]**○Kazuhiro Matsuo¹, Kunihiro Okamoto¹, Yasuo Noda¹, Hideki Tachibana², Shin-ichi Segawa¹¹Sch. Sci. & Tech., Kwansei Gakuin Univ., ²Fac. Sci., Kobe Univ.

Glycerol is well known as a solvent additive to stabilize protein. At which stage does it work effectively to regenerate the native disulfide bonds of lysozyme? We studied the regeneration process of 2SS-variant of lysozyme, 2SS[6-127, 64-80]. This sample is suitable for our study, because the number of intermediates produced during the disulfide regeneration is limited and its partially ordered structure is likely to be recovered by the addition of glycerol judging from its CD spectra. Three peaks of 2SS-intermediates and a single peak of 1SS-intermediate and a single peak of fully reduced species were clearly separated on a C4-HPLC column. At first, the regeneration reaction was carried out at 0.1 mM GSSG (oxidized glutathione) in the absence of GSH (reduced glutathione). Irrespective of whether glycerol was added or not, the amount of fully reduced species decreased at the same rate and native 2SS species also appeared at a similar rate. Further, non-native 2SS-intermediates were significantly populated. On the other hand, under the regeneration condition of 0.1 mM GSSG and 1 mM GSH, enough amount of 1SS-intermediates and non-native 2SS-species remained even after 32 min in the absence of glycerol, but 1SS-intermediates almost disappeared, and non-native 2SS-species existed only slightly in the presence of 30% glycerol. These data indicate that glycerol does not contribute to the stabilization of either 1SS-intermediates or non-native 2SS-species, but greatly contribute to the stabilization of native 2SS-species folded correctly.

1P128**Role of an off-barrel strand for the structural stabilization of equine β -lactoglobulin**

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β -Lactoglobulin is a major component of mammalian milk and a member of the lipocalin family. The members of this family share an eight-stranded (labeled A-H) up-and-down β -barrel structure with an α -helix and an off-barrel strand I. The α -helix makes a tight hydrophobic packing with the strand H and G, The C-terminus of the strand A is sandwiched between the off-barrel strand I and strand H, forming a β -sheet structure. The β -structure made by the strand I, A, H seems to have an important role in the stabilizing the overall β -barrel structure. To investigate that the role of an off-barrel strand for the structural stabilization of equine β -lactoglobulin (ELG), we made two mutant proteins in which the C-terminal 10 residues or 20 residues were removed (152 Δ and 142 Δ , respectively). At pH 7.0, the circular dichroism (CD) spectrum of 152 Δ is similar to that of wild-type ELG (WT). In a heteronuclear single quantum coherence (HSQC) spectrum of 152 Δ , the cross-peaks are well dispersed along both ¹H and ¹⁵N axes. These results indicated that 152 Δ forms a stable native-like structure. However, the CD and HSQC spectrum have shown that the 142 Δ can not acquire any native-like structure under physiological condition. Therefore, the off-barrel strand is important for maintaining the β -barrel structure.