ON THE RENATURATION OF REDUCED HEN EGG WHITE LYSOZYME CONTAINING TWO BLOCKED SULFHYDRYL GROUPS

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Formation of native lysozyme from the reduced form involves many pathways in two processes: incorrect pairing of half-cystine residues by oxidation and rearrangement of disulfide (SS) bonds (1). The energy barrier against suflhydryl (SH)-disulfide interchange of the native or nativelike species thus formed causes accumulation of these species (1, 2). For example, the enzymatically active isomers containing three presumably native SS bonds and one open SS bond may be thermodynamically favorable over the nonnative isomers and can be formed from reduced lysozyme or lysozyme containing "scrambled" SS bonds by nonobligatory and flexible pathways (1,3–5). As an extension of these observations formation of nativelike species from reduced lysozyme containing the average of two carboxymethyl (CM)-cysteine was investigated.

Reduced lysozyme was reacted with 2 mol of [1-^{14}C]iodoacetic acid in the presence of 6 M guanidine HCl (3). The reduced, partially [^{14}C]carboxymethylated lysozyme (25 μg/ml) thus obtained was air-oxidized in the presence of 1.5 mM β-mercaptoethanol at pH 7.8 for 24 h at 23°C (3). Gel filtration (1) of the soluble products (15%) resulted in separation of the higher and the lower hydrodynamic (LH) volume forms containing average 2.3 and 1.3 mol, respectively of the ^{14}C label in yields of 15 and 85%, respectively. Gel-filtration of the LH forms followed by ion exchange chromatography at pH 7.0 (5) yielded samples, A, B, C, D, and E (in the order of elution) in 2.6, 8, 30, 34, and 24%, respectively. Samples B and C contained ~2 mol of the ^{14}C label and exhibited 12–15% enzymatic activity of native lysozyme. Sample D contained 1.2 mol of the ^{14}C label and exhibited 35% enzymatic activity. Sample E was nonradioactive and exhibited 100% enzymatic activity. By polyacrylamide gel electrophoresis, samples B and C exhibited the same mobility as that of a standard dicarboxymethylated sample (4, 5). On analysis of tryptic peptides containing the ^{14}C label (1) 33, 22, and 45% radioactivity of sample B were found with peptide T_3 (Cys 6), T_{17+18} (Cys 127) and T_{11} (Cys 76, 80, and 94), respectively, and 20, 26, 44, and 11% radioactivity of sample C with peptide T_3, T_{17+18}, T_{11}, and T_{15} (Cys 115), respectively.

Sample E was apparently noncarboxymethylated, renatured lysozyme and sample D monocarboxymethylated species containing three presumably native SS bonds (3–5). Samples B and C were dicarboxymethylated. Completely random carboxymethylation of reduced lysozyme with 2 mol of iodoacetate would result in dicarboxymethylation of 31.1% population by probability. Only 4 of 28 isomers of dicarboxymethylated, reduced lysozyme could form three native SS bonds. Then, a maximum yield of these four isomers would be 4.4% which is close to the overall yield of sample B plus C (4.8%). However, since no radioactivity was found with peptide T_6 (Cys 30) and T_9 (Cys 64) (1) in both cases, sample B and C could contain only two such isomers dicarboxymethylated at position 6 and 127, or at position 76 and 94 (6) (a maximum yield, 2.2%). Thus, to account for the yield of sample B

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plus C, which is >2.2%, dicarboxymethylated reduced lysozyme would have to be assumed to form the species with two native SS bonds and two open SS bonds, for example, between Cys 6 and Cys 127 (one CM-cysteine and one SH) and between Cys 76 and Cys 94 (one CM-cysteine and one SH). Indeed, sample C was found to contain 1.6 mol of free SH groups as determined by the method described earlier (5). Further support of this hypothesis is obtained as follows.

After similar incubation (37°C) of the reduced, partially [^{14}C]carboxymethylated lysozyme (see above), the products were treated with 10 fold molar excess (over β-mercaptoethanol) of cold iodoacetic acid in the presence of 4 M urea. Ion exchange chromatography of the LH forms yielded an enzymatically active (12% activity) containing 2 mol of the ^{14}C label and exhibiting the same electrophoretic mobility as that of a standard tetracarboxymethylated lysozyme (7) (overall yield, 1.4%). The radioactivity of this sample was found only with peptide T_3, T_{17+18}, and T_{11} in 30, 40, and 30% yields, respectively. Completely random oxidation of the reduced lysozyme containing the average of two CM-cysteine would yield, in theory in 0.9% (31.1% × 36/1,260) population, 36 possible isomers containing two native SS bonds, two CM-cysteine, and two SH groups. However, not all these isomers appeared to be present (in the tetracarboxymethylated form) in the isolated LH form. Then, it would follow that the isolated isomers may be thermodynamically favorable ones. These observations are also consistent with that an enzymatically active (15%) derivative containing two presumably native SS bonds and four CM-cysteine at position 6, 76, 94, and 127 exhibits a native fold and is considerably stable in the presence of 1.5 mM β-mercaptoethanol (7) and suggest that not all isomers containing native SS bonds and SH groups may be of equivalent stability until more than two native SS bonds are formed (3, 4).

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