SECONDARY STRUCTURE OF REDUCED OVOMUCOID AND RENATURATION OF REDUCED OVOMUCOID AND ITS REDUCED FRAGMENTS A(1–130) AND B(131–186)

Tsukasa MATSUDA, Kenji WATANABE and Yasushi SATO
Faculty of Agriculture, Nagoya University, Chikusa-ku, Nagoya 464, Japan

Received 10 November 1980

1. Introduction

Chicken ovomucoid is a trypsin inhibitor, which has 9 disulfide bridges, no sulfhydryl group, and 185 amino acid residues [1]. The amino acid sequence of chicken ovomucoid has been determined to consist of 3 separated domains, each being homologous and containing 3 intradomain and no interdomain disulfide bridges [1]. This characteristic sequence has been said to result from gene elongation by duplication [1,2].

It was reported that reduced turkey ovomucoid had no trypsin and chymotrypsin inhibitory activities but >90% of the activities were regained upon reoxidation [3]. Refolding of the other disulfide-containing proteins to the native conformation from the unfolded state in reducing and denaturing reagents has been investigated mainly with bovine ribonuclease (RNase) and bovine pancreatic trypsin inhibitor (BPTI) [4–8]. The results of these investigations have suggested that fully reduced proteins have little or no ordered conformation [9–11]. However, the problem of residual native structures in reduced proteins has not been completely elucidated. It was also reported that the entire amino acid sequence of proteins was essential to determine the unique folding [12,13]. In view of the characteristic amino acid sequence above, ovomucoid domains are likely to fold individually after they are cut apart.

This communication presents the results of experiments designed to estimate the secondary structure content of reduced chicken ovomucoid before the reformation of disulfide bonds and to test whether ovomucoid fragments A (residue no. 1–130) and B* (residue no. 131–186) can form a correct conformation after reduction and reoxidation.

2. Materials and methods

Ovomucoid was isolated from chicken egg white [14], fragments A and B were prepared from ovomucoid by *Staphylococcus aureus* V-8 protease digestion [2], and their homogeneities were confirmed as in [15].

Redistilled water was used throughout. Tris–hydroxymethylaminoethane (Tris) was recrystallized from 90% methanol. β-Mercaptoethanol and guanidine–HCl were ultrapure grade and copper sulfate was reagent grade. These were used without further purification.

Ovomucoid and its fragments were reduced and reoxidized essentially as in [16]. Ovomucoid or its fragments (5–10 mg) were reduced by treatment with 0.3 M mercaptoethanol in 6 M guanidine–HCl–0.01 M EDTA–0.025 M Tris–HCl buffer (pH 8.0) for 24 h at 30°C to yield a randomly coiled polypeptide chain. The reduced samples were separated from the reagents by passage through a column (2.0 × 25 cm) of Sephadex G-75 equilibrated with 0.025 M Tris–HCl buffer (pH 8.0). In order to prevent heavy-metal ions from contaminating the reduced samples, all vessels, measuring cells and tubes were carefully washed with 0.01 M EDTA and rinsed with redistilled water. The buffer for the elution was saturated with nitrogen gas. A part of reduced samples was kept at 4°C in a stoppered tube filled with nitrogen gas and the other part, to which 10^{-7} M $CuSO_4$ was added,

* There are two kinds of fragment B, either with or without carbohydrate [1,2]; that containing carbohydrate was used here

Address correspondence to K. W.

was reoxidized at 25°C by shaking gently in the air. In some experiments, guanidine–HCl was added to 6 M to the reduced ovomucoid solution.

Circular dichroism (CD) spectra of samples were measured at 25°C with a JASCO J-40 CS spectropolarimeter, and mean residue ellipticities $[\theta]$ were calculated on the basis of average residue weights. All of the spectra were corrected by subtracting the spectra of carbohydrate moiety as in [15].

Sulfhydryl analysis was done as in [17] in 6 M guanidine–HCl. Protein concentration was determined as in [18]. Trypsin inhibitory activity was assayed by measuring the initial rate of increase in A_{420} with α-*N*-benzoyl–L-arginine *p*-nitroanalide as in [14].

3. Results

As seen in fig.1, the CD spectrum of reduced ovomucoid showed intermediate shape and intensities between those of native ovomucoid in the Tris–HCl buffer and of reduced ovomucoid in 6 M guanidine–HCl immediately after preparation, and did not change even after keeping under nitrogen gas for 8 h. The ovomucoid CD spectrum changed by the reduction of disulfide bonds was gradually restored toward that of native ovomucoid. The spectrum of ovomucoid reoxidized for 24 h had fully recovered its original shape and intensities.

The secondary structure compositions of reduced and reduced–reoxidized ovomucoids, which were computed from their CD spectra as in [19], are shown in table 1. The coincidence between the experimental and calculated CD spectra for the reduced ovomucoid, as well as for the native one [15], was satisfactory between 212 and 240 nm as seen as in fig.1. Both α-helix and β-structure contents in reduced ovomucoid were ~60% of those in native ovomucoid,

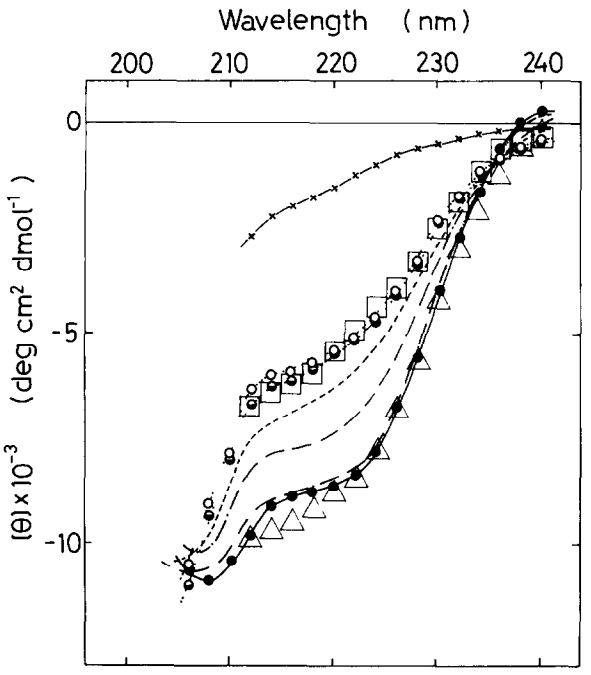

Fig.1. CD spectra of reduced and reduced–reoxidized ovomucoids. The CD spectra of reduced ovomucoid were measured immediately after preparation (· ·○· ·) and after keeping for 8 h under nitrogen gas (· ·●· ·). The CD spectra of reduced–reoxidized ovomucoid were measured after reoxidation for 2 h (– –) 7 h (–.–) and 24 h (–. .–). The CD spectra of reduced ovomucoid in 6 M guanidine–HCl (–x–) and native ovomucoid in Tris–HCl (pH 8.0) (–●–) were also measured for comparison. The calculated CD spectra for the native (△) and reduced (□) ovomucoids were obtained by substituting the calculated secondary structure compositions into the equation in [19].

and they were gradually recovered as the reoxidation proceeded.

Reduced ovomucoid had no trypsin-inhibitory activity, but the activity was regained upon reoxidation, and >95% of its original activity was observed

Table 1
Secondary structure compositions of reduced and reduced–reoxidized ovomucoid

Samples	Secondary structure composition (%)			
	α-Helix	β-Structure	β-Turn	Random coil
Native ovomucoid	26	46	10	18
Reduced ovomucoid	16	34	20	30
Reduced–reoxidized ovomucoid	23	48	8	21

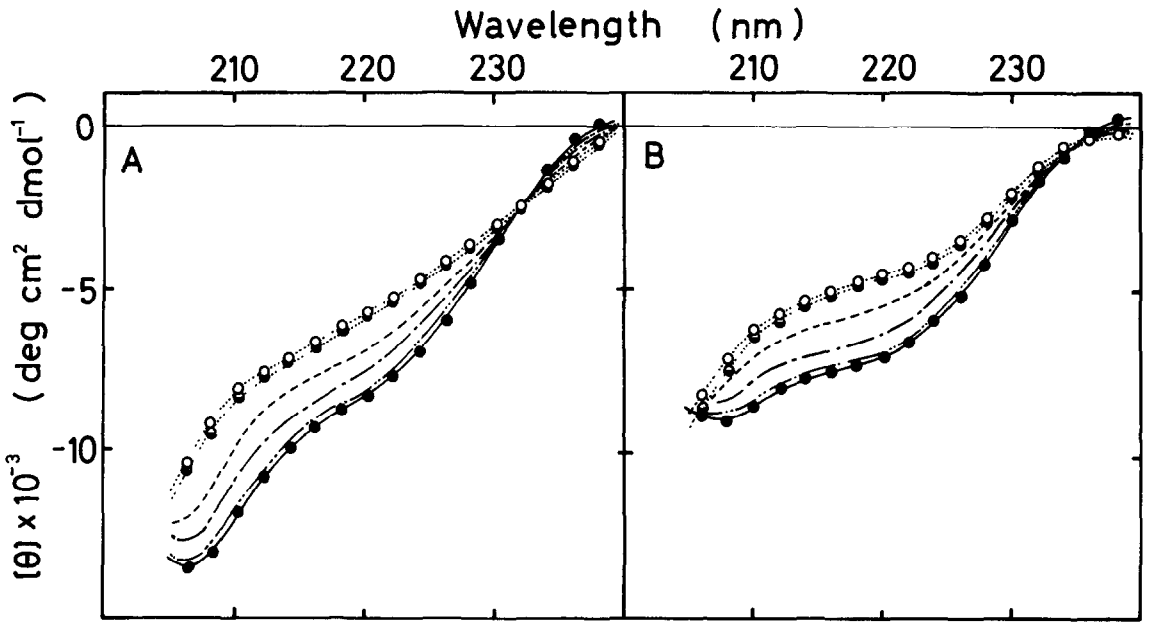

Fig.2. CD spectra of reduced and reduced–reoxidized fragments A and B. Conditions and symbols are the same as indicated in fig.1. (A) fragment A; (B) fragment B.

after reoxidation for 24 h. The sulfhydryl content in reduced ovomucoid was 18.4 and 17.8 groups/molecule, respectively, immediately after the preparation and after keeping under nitrogen gas for 8 h. The same values were obtained within experimental error after the measurement of CD spectra. On the other hand, in the presence of copper ion, the sulfhydryl groups in reduced ovomucoid were gradually decreased and they were not detected after reoxidation for 24 h.

The CD spectra of reduced and reduced–reoxidized fragments A and B are shown in fig.2. Both reduced fragments showed the CD spectra with a considerable intensity of negative ellipticities, and the spectra were fully restored upon reoxidation in a manner similar to ovomucoid.

The fragment A, which consists of domains I and II, showed trypsin inhibitory activity nearly equal in mol to that of ovomucoid, but the fragment B, or domain III, showed no such activity [1,2]. The reduced fragment A had no detectable activity, but the activity was gradually regained upon reoxidation. The activity of reduced–reoxidized fragment A was >98% of its original level. The sulfhydryl contents in reduced fragments A and B were 11.2 and 6.7 groups/peptide, respectively (the fragments A and B contain 6 and 3 cystine residues, respectively [1,2]).

4. Discussion

The method of Cu^{2+}-catalyzed air oxidation was used in this experiment simply because disulfide reagents contribute to CD spectra [20], though more satisfactory procedures for reoxidation with disulfide reagents have been introduced [21].

The reduced BPTI was assumed to have a disordered conformation in solution from its absence of inhibitory activity and its relative insolubility [22]. However, measurement of the CD spectra of reduced RNase, which differed from that of native protein, [10,11], suggested that the reduced RNase did not completely lose its ordered conformation. Moreover, the ORD properties of several enzymes have indicated that the secondary structure was formed before the final conformation was attained [23].

Protein secondary structure has been said to be important as nucleation centres at the initial stage of protein folding [24,25]. It was also proposed that the sections with β-turn propensities in a protein must play a role in bringing distant parts of polypeptide chain together [26,27]. It is therefore likely that the secondary structure was formed in the reduced ovomucoid before the reformation of disulfide bonds, though it is uncertain whether the secondary structure formed in the initial stage of folding remained unchanged.

Though a certain degree of uncertainty derived from the least-squares method used for the calculation of secondary structure as well as from the reference spectra in [18], it was concluded from the estimated secondary structure of reduced ovomucoid that the reduced ovomucoid had already recovered a certain degree of secondary structure before the formation of disulfide bonds. Moreover, little change of CD spectrum of reduced ovomucoid by keeping under the condition to prevent reoxidation suggested that the partial refolding of reduced ovomucoid to this stage from the unfolding state proceeded as soon as the denaturants had been removed, and that the reformation of disulfide bonds was a necessary requirement for further refolding to occur.

The domains of some avian ovomucoids containing 2 or 3 reactive sites were reported to retain their inhibitory activities individually even after they were cut apart [1,2,28]. In this experiment as well, fragment A fully retained the trypsin inhibitory activity. Moreover, the reconstituted fraction of ovomucoid secondary structure calculated from the CD spectra of fragment A and B was in good agreement with the fraction estimated from the ovomucoid CD spectrum in [15]. Therefore, it is reasonable to say that the conformations of fragments A and B were the same as in the intact ovomucoid.

The refolding of fragment A indicated that the removal of the 56 residues of carboxyl end did not cause destruction of the information to form the correct conformation of domains I and II upon reoxidation. In addition, the recovery of CD spectrum of reduced–reoxidized fragment B suggested that domain III could refold independently of the other two domains. The reduced fragments of RNase [12] and BPTI [13] lacking the several C-terminal residues formed a random mixture of disulfide bonds under refolding condition where normal proteins refold correctly. However, it is presumed that ovomucoid originally consisted of only 1 domain and had been converted into a 2-domain ovomucoid and further into a 3-domain ovomucoid by gene duplication resulting from unequal crossing-over [1,2]. If this presumption is true, it is natural that each domain in the ovomucoid has the individual information to form a unique, stable and functional structure. It was concluded that each domain of ovomucoid could refold individually after separation by hydrolysis of the connection peptides and that the ovomucoid conformation consisted of the 3 structural domains corresponding to the domains on the amino acid sequence.

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