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Position of the Disulfide Bond in Ovalbumins of Differing Heat Stability. Elimination of Thiol–Disulfide Interchange as a Mechanism for the Formation of the Ovalbumins

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Abstract

Peptides containing the four cysteine and two half-cystine residues labelled with [2-^{14}C]iodoacetic acid were isolated from thermolytic digests of reduced and *S*-carboxymethylated ovalbumin by paper ionophoresis, pH 6·4, descending paper chromatography and another ionophoresis at pH 1·9. These peptides were analysed for amino acids and the peptides identified with their position in the known linear sequence of the molecule.

The location of the disulfide bond for three ovalbumins of differing heat stability were determined by blocking the cysteine residues with non-radioactive iodoacetic acid, reducing the disulfide bond and labelling the half-cystine residues with [2-^{14}C]iodoacetic acid. Radioactive peptides were isolated after thermolytic digestion by paper ionophoresis and descending paper chromatography. The amount of radioactivity on each peptide was quantitated and the two half-cystine labelled peptides accounted for over 90% of radioactivity.

The disulfide bond was found to involve the sequences:

Phe-Gly-Asp-SerP-Ile-Glu-Ala-Gln-Cys-Gly-Thr-Ser

and

Leu-Gln-Cys

for the three ovalbumins of differing heat stability. Therefore thiol–disulfide interchange is not involved in the formation of these ovalbumins.

Introduction

Ovalbumin is a glycoprotein which contains four cysteine residues and one disulfide bond (Fothergill and Fothergill 1970). Its messenger RNA sequence and hence its amino acid sequence was recently determined by McReynolds *et al.* (1978) and the position of the disulfide allocated to the third and fourth half-cystine residues from the amino terminal by Thompson and Fisher (1978).

Subsequent to laying, the pH of egg white rises from 7·6 to 9·0 as CO_2 is lost through the shell (Smith 1969). Accompanying this change is an increase in resistance to heat denaturation. Smith (1964) isolated a more stable form of ovalbumin which he called S-ovalbumin and later Donovan and Mapes (1976) found that S-ovalbumin formation proceeds through an intermediate species. These three ovalbumin forms are designated as ovalbumin, S1- and S2-ovalbumin in order of increasing resistance to heat denaturation.

The specific structural difference between the three ovalbumins has not been determined. Smith and Back (1965), however, have shown that the conversion does not involve the loss of single amino acids or the loss of a peptide as occurs during the plakalbumin transformation. A small conformational change does

accompany the conversion and recent work by Kint and Tomimatsu (1979) has indicated that 3–4% of the ovalbumin molecule changes from an α-helix to an anti-parallel β-sheet configuration.

It has been suggested by Smith and Back (1968*a*) that the formation of a more heat-stable form of ovalbumin may involve a change in covalent bond structure. As the conversion is pH-dependent and in the range of ionization of thiol groups, which are known to catalyse thiol–disulfide interchange in alkaline solutions (Ryle and Sanger 1955), an interchange mechanism may explain the conversion of native ovalbumin to more thermodynamically stable forms.

The aim of the present study was to identify the half-cystine residues involved in the disulfide bond of ovalbumins of differing heat stability and to quantitate the extent of thiol–disulfide interchange during their formation.

Materials and Methods

Preparation of Ovalbumin, S1- and S2-ovalbumin

Native ovalbumin was prepared from freshly laid hen's eggs by recrystallization as described by Warner (1954). Conversion of native ovalbumin to more heat-stable forms followed the method of Smith and Back (1965). The protein (2 g) was dissolved in 40 ml water, the pH adjusted to 10·0 with 0·1 M NaOH and two 20-ml (1 g) aliquots heated at 55°C for 5 and 30 h. After cooling to room temperature the pH was lowered to 4·7 with 0·1 M HCl, the solution centrifuged to remove denatured protein, dialysed and freeze-dried.

Scanning Calorimetry

The S-ovalbumin content of the three ovalbumin preparations was determined by differential scanning calorimetry. The temperature of the maximum rate of denaturation was measured in the adiabatic scanning calorimeter described by Smith and Rose (1975). A 1 mM protein solution was made up in 0·05 M phosphate buffer (pH 7·0) and the scanning rate was 25°C per hour.

Preparation of Labelled SCM-ovalbumins

Labelling of cysteine and half-cystine residues

Labelled [2-^{14}C]iodoacetic acid (57 mCi/mmol) was supplied by the Radiochemical Centre, Amersham, England. It was dissolved in water and stored frozen (500 μCi/ml).

Native ovalbumin (100 mg) was dissolved in 1·0 ml distilled and de-ionized water (N.B.: all solutions were flushed with nitrogen for a minimum of 20 min prior to use), mixed with 4·0 ml 10 M urea in 1 M Tris-HCl–20 mM EDTA, pH 10·0, and 1·6 mg dithiothreitol (10-fold thiol groups per disulfide bond). The reaction flask containing the mixture at pH 10·0 was flushed with nitrogen and kept at 37°C for 16 h. Then the pH was lowered to 8·5 with 2 M HCl, 100 μl (50 μCi) of radioactive [2-^{14}C]iodoacetic acid and 2·28 mg of non-radioactive iodoacetic acid (1·1-fold molar excess over protein –SH) added. After flushing the reaction flask with nitrogen and incubating at 37°C for 20 min in the dark alkylation was completed with 50 mg non-radioactive iodoacetic acid and 40 mg Tris dissolved in 500 μl 8 M urea in 1 M Tris-HCl–20 mM EDTA, pH 8·5, under nitrogen for 20 min at 37°C in the dark. Isolation of labelled SCM-ovalbumin followed the method of Smith and Back (1968*b*). The pH of the solution was lowered to 4·8 with 17 M acetic acid, 30 ml water added and the precipitate was removed by centrifugation. Washing of the precipitate was repeated twice and the final precipitate freeze-dried.

Labelling of disulfide-linked half-cystine residues

For the labelling of the disulfide bond, ovalbumin (100 mg) was dissolved in 1·0 ml water and centrifuged to remove any denatured protein. To the protein solution 4 ml 10 M urea–0·1 M HCl–20 mM EDTA (pH of the solution approximately 2·5) with 100 mg of non-radioactive iodoacetic acid was added with stirring. After 60 s 500 μl 3 M Tris with 100 mg non-radioactive iodoacetic acid (total iodoacetic acid 135-fold excess over cysteine) titrated to pH 8·5 with 10 M NaOH was

added with stirring, the reaction flask flushed with nitrogen and incubated at 37°C for 20 min in the dark. The protein with cysteine-blocked residues was recovered as previously described but the final precipitate was taken up and dissolved in 0·5 ml 1 M Tris-HCl–20 mM EDTA, pH 10·0. 4 ml 10 M urea–1 M Tris-HCl–20 mM EDTA, pH 10·0, and 1·6 mg dithiothreitol was added to the protein solution, the reaction flask flushed with nitrogen and the reduction mixture kept at 37°C for 16 h. Then the pH was lowered to 8·5 with 1 M HCl, 100 μl of radioactive [2-^{14}C]iodoacetic acid and 0·65 mg non-radioactive iodoacetic acid (1·1-fold molar excess over protein-SH) added and the reaction flask flushed with nitrogen. After 20 min at 37°C in the dark alkylation was completed as outlined above. The SCM-ovalbumin with labelled half-cystine residues was recovered as previously described and the final precipitate freeze-dried or immediately digested.

Enzyme Digestion

Thermolysin digestion was carried out in 1% (w/v) ammonium bicarbonate (pH 8·0) at 37°C for 16 h using 3% enzyme on the weight of substrate. The digest was freeze-dried three times using 10% (v/v) isopropanol as solvent.

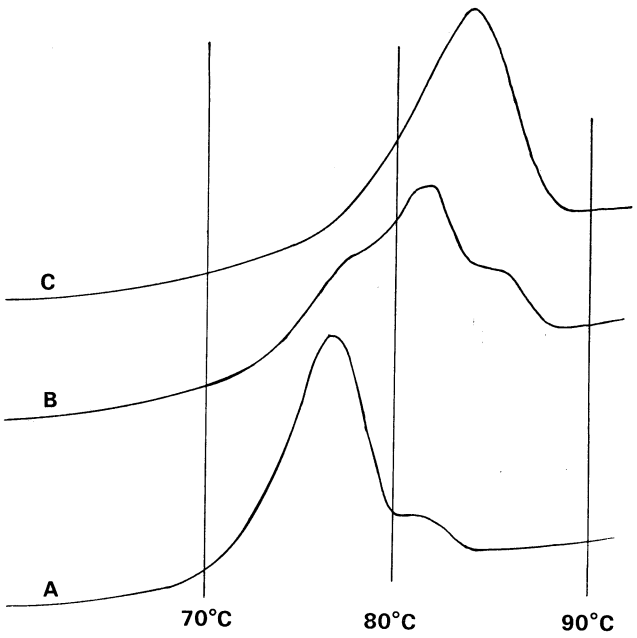

Fig. 1. Representative curves (thermograms) of differential heating rate versus temperature for three ovalbumin preparations. *A*, Ovalbumin. *B*, S1-ovalbumin. *C*, S2-ovalbumin. Scanning rate 25°C per hour.

Peptide Fractionation and Identification

Peptides were separated by paper ionophoresis at pH 6·5, descending paper chromatography in butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12 v/v) and paper ionophoresis at pH 1·9.

Radioactive peptides were detected by autoradiography and eluted from the paper with 6 M HCl. Mercaptoethanol (10 μl of a 5% v/v solution) was added and the hydrolysis carried out in a sealed evacuated tube at 110°C for 20 h. Amino acid analysis was performed either by running the hydrolysate on a Beckman amino acid analyser, model 121 M, or by paper chromatography at pH 1·9. Amino acids were detected by 0·2% (w/v) ninhydrin, 5% (v/v) acetic acid and 2·5% (v/v) dicyclohexylamine in ethanol.

Measurement of Radioactivity

Radioactive peptides were exhaustively eluted from the paper using a glass modification of the method of Edstrom (1968) with 60% (v/v) pyridine and dried down under a stream of nitrogen.

A mixture of toluene phosphor–Triton X–methanol (20 : 15 : 1 v/v) was used for liquid scintillation counting. The toluene phosphor mixture contained 8 g 2,5-diphenyloxazole (PPO) and 50 mg *p*-bis-(5-phenyloxazol-2-yl)-benzene (POPOP) in 1 litre toluene.

Performic Acid Oxidation

Isolated peptides were dissolved in 1·0 ml of performic acid preformed from 30% (v/v) H_2O_2 (0·5 ml) and 98–100% (v/v) formic acid (9·5 ml) by standing at room temperature for 1 h. After 1 h at 37°C the solution was diluted with 19·0 ml water and freeze-dried twice.

Results

Formation of Ovalbumin, S1- and S2-ovalbumin

Three ovalbumin preparations were heated at 55°C, pH 10·0, for 0, 5 and 30 h. Rate of denaturation curves (thermograms) were prepared using an adiabatic scanning calorimeter (Fig. 1) and the S-ovalbumin content of each preparation

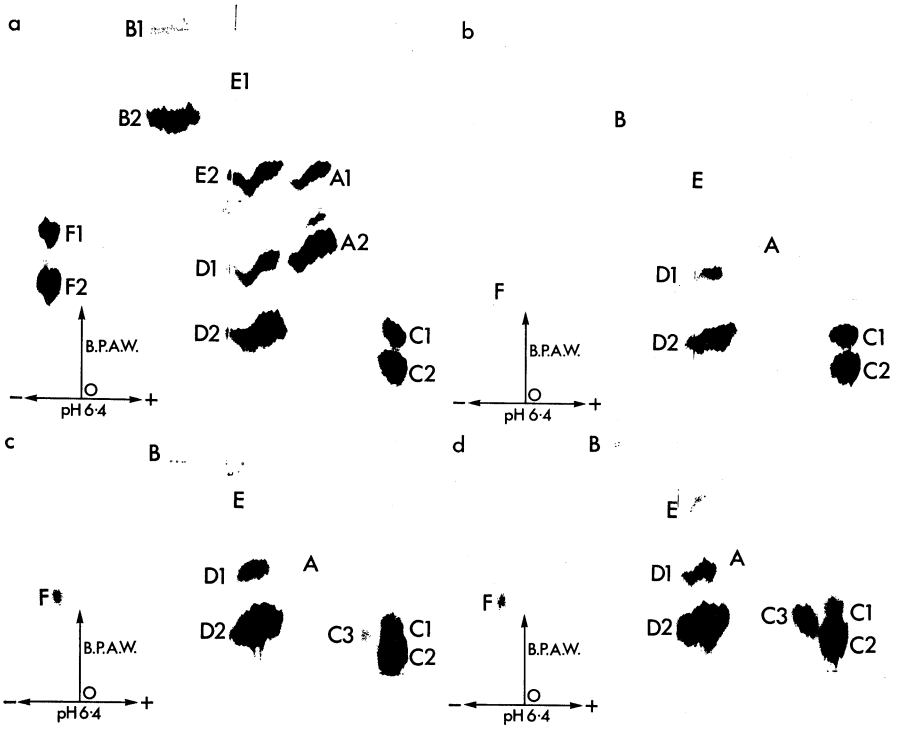

Fig. 2. Autoradiograms of the radioactive zones of (*a*) [2-^{14}C]carboxymethyl half-cystine plus carboxymethylcysteine and (*b–d*) [2-^{14}C]carboxymethyl half-cystine peptides of ovalbumin after separation by paper ionophoresis, pH 6·4, and paper chromatography with butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12 v/v). The zones are labelled A–F to designate sequences as outlined in the text. (*a*), (*b*) Ovalbumin; (*c*), S1-ovalbumin; (*d*) S2-ovalbumin.

estimated. Native ovalbumin (0 h) is not homogeneous, it has a maximal rate of denaturation peak at 76·5°C and a minor peak at 81·5°C representing less than 5% S1-ovalbumin contamination. The S1-ovalbumin preparation (5 h) is the least homogeneous of the three as it is a transient species between ovalbumin (25%) and S2-ovalbumin (15%). Conversion to S2-ovalbumin is essentially complete after 30 h of the conversion regime (maximal rate of denaturation at 85°C).

Separation of the Labelled Carboxymethylcysteine Peptides

The initial separation of a thermolytic digest of reduced and labelled SCM-ovalbumin is shown in Fig. 2*a*. Peptides were fractionated by paper ionophoresis at pH 6·5 followed by descending paper chromatography. Each radioactive zone was further purified by paper ionophoresis at pH 1·9.

Peptide Analysis

Peptides eluted from the paper were either an *S*-carboxymethylcysteine containing peptide or its sulfoxide probably formed due to oxidation by pyridine oxides in the fractionation buffers. The oxidation state of a peptide was identified by whether the peptide underwent further oxidation upon rechromatography. If it did not the peptide was in the stable sulfoxide form. A comparison with the *S*-carboxymethylcysteine sulfone peptide formed by performic acid oxidation at 37°C (Takahashi 1973) and with the original peptide as shown in Fig. 3 was helpful.

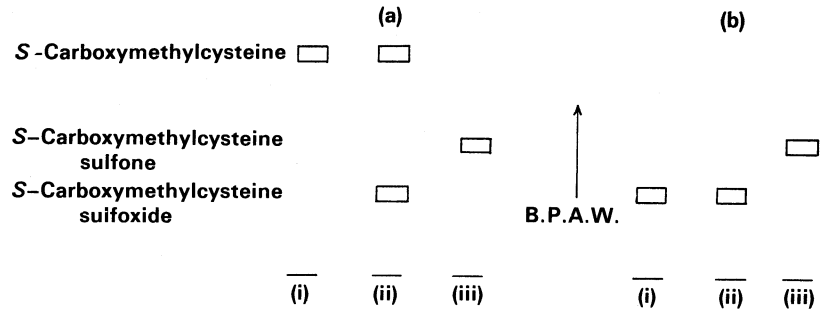

Fig. 3. Composite diagram showing the relative rates of movement of radioactive zones of peptide D1 (*a*) and peptide D2 (*b*). For (*a*): (i) *S*-carboxymethylcysteine form; (ii) *S*-carboxymethylcysteine form re-run in butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12 v/v); (iii) *S*-carboxymethylcysteine sulfone form. For (*b*): (i) *S*-carboxymethylcysteine sulfoxide form; (ii) *S*-carboxymethylcysteine sulfoxide form re-run in butanol–pyridine–acetic acid–water as above; (iii) *S*-carboxymethylcysteine sulfone form.

After elution from the paper peptides containing *S*-carboxymethylcysteine presumably underwent extensive oxidation to the sulfoxide. It is probable that the sulfoxide decomposed to cysteine during hydrolysis (Takahashi 1973) with the formation of cystine and cysteic acid if traces of oxygen were present. The formation of cysteic acid would cease when all available oxygen was utilized and this would explain the low yield of cysteic acid obtained after hydrolysis. Thompson and Fisher (1978) hydrolysed their samples in unevacuated tubes without a thiol scavenger in the presence of abundant oxygen ensuring more complete oxidation of cysteine and cystine to cysteic acid (Moore and Stein 1963) giving a high quantitative return. The inability to resolve cysteine and cystine quantitatively on the amino acid analyser resulted in the low yield of products derived from cysteine reported here.

The sequence around the cysteine and half-cystine residues independently arrived at by Thompson and Fisher (1978) and McReynolds *et al.* (1978) were used together with the amino acid compositions of the thermolytic peptides to place them in their order A–F in the linear sequence. Their amino acid composition, sequence

Table 1. Amino acid composition, sequence and oxidation state of [2-^{14}C]carboxymethylcysteine plus carboxymethyl half-cystine thermolytic peptides of ovalbumin

Peptide	Amino acid composition	Amino acid sequence
A1	$Phe_{1.0}CySO_3H_{+ve}$	Phe-CMCys
A2	$Phe_{1.0}CySO_3H_{+ve}$	Phe-CMCySO[A]
B1	$Phe_{1.0}Tyr_{0.8}CySO_3H_{0.2}Pro_{1.0}Ile_{1.2}$	Phe-Tyr-CMCys-Pro-Ile
B2	$Phe_{1.0}Tyr_{0.9}CySO_3H_{0.3}Pro_{0.9}Ile_{1.2}$	Phe-Tyr-CMCySO-Pro-Ile
C1	$Phe_{0.8}Gly_{2.2}Asp_{1.0}Ser_{1.6}Ile_{0.6}Glu_{2.0}Ala_{1.0}CySO_3H_{0.4}Thr_{0.9}$	Phe-Gly-Asp-SerP-Ile-Glu-Ala-Gln-CMCys-Gly-Thr-Ser
C2	$Phe_{0.8}Gly_{2.2}Asp_{1.0}Ser_{1.7}Ile_{0.6}Glu_{1.8}Ala_{1.0}CySO_3H_{0.2}Thr_{0.8}$	Phe-Gly-Asp-SerP-Ile-Glu-Ala-Gln-CMCySO-Gly-Thr-Ser
C3	$Ala_{1.0}Glu_{1.0}CySO_3H_{0.2}Gly_{1.3}Thr_{0.8}Ser_{0.9}$	Ala-Gln-CMCySO-Gly-Thr-Ser
D1	$Leu_{1.0}Glu_{1.0}CySO_3H_{0.3}$	Leu-Gln-CMCys
D2	$Leu_{1.0}Glu_{1.2}CySO_3H_{0.1}$	Leu-Gln-CMCySO
E1	$Leu_{1.0}Phe_{1.0}CySO_3H_{0.2}$	Leu-Phe-CMCys
E2	$Leu_{0.9}Phe_{1.0}CySO_3H_{0.2}$	Leu-Phe-CMCySO
F1	$Phe_{1.0}Gly_{1.4}Arg_{1.1}CySO_3H_{0.6}$	Phe-Gly-Arg-CMCys
F2	$Phe_{1.0}Gly_{1.1}Arg_{1.0}CySO_3H_{0.4}$	Phe-Gly-Arg-CMCySO

[A] CMCySO is an abbreviation for *S*-carboxymethylcysteine sulfoxide.

and oxidation state are listed in Table 1 and the distribution of radioactivity on each peptide A–F is shown in Fig. 4*a*).

Disulfide-linked Half-cystine Peptides of Ovalbumins of Differing Heat Stability

Ovalbumin

The separation of the labelled disulfide-linked half-cystine peptides of native ovalbumin is shown in Fig. 2*b* and the distribution of radioactivity on each peptide A–F shown in Fig. 4*b*. Peptides C and D account for 94·7% of the total radioactivity between them with 3·6% accounted for by the remaining four peptides and 1·7% due to miscellaneous unidentified radioactive zones.

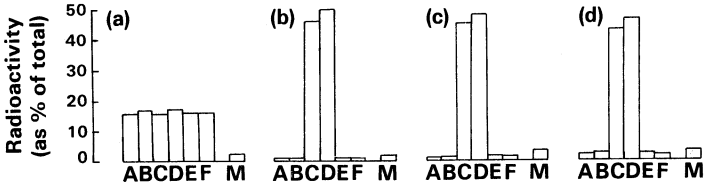

Fig. 4. Percentage of total radioactivity on the (*a*) [2-^{14}C]carboxymethyl half-cystine plus carboxymethylcysteine and (*b–d*) [2-^{14}C]carboxymethyl half-cystine peptides of ovalbumin. Columns A–F designate peptide sequences as outlined in the text. M denotes any miscellaneous radioactive zones not identified. (*a*), (*b*) Ovalbumin; (*c*) S1-ovalbumin; (*d*) S2-ovalbumin.

When a 1·1-fold molar excess of iodoacetic acid or iodoacetamide is used during carboxymethylation of the four cysteine plus the reduced two half-cystine residues or the two reduced disulfide-linked half-cystine residues, a uniform distribution of radioactivity occurs over the six and two thermolytic peptides respectively. But when [2-^{14}C]iodoacetic acid is used undiluted with carrier and in non-stoichiometric amount the distribution of radioactivity is not uniform. The C peptide characteristically possesses a lower total radioactivity and this was especially significant during disulfide labelling in which up to 20% difference in radioactivity between the C and D peptides was observed in some experiments, even though the two of them still accounted for more than 90% of the total radioactivity. This effect appears to be charge-related and may be due to the close proximity in primary sequence C of a phosphorylated serine residue.

Thiol–disulfide interchange is catalysed by thiol groups in alkaline solutions (Ryle and Sanger 1955). Under conditions of denaturation in 8 M urea (pH 8·5) and blocking of cysteine with a small excess of iodoacetic acid, a common procedure for many workers, particularly when radioactive reagent is used to give a high specific activity (for example, Fothergill and Fothergill 1970; McKenzie *et al.* 1972, fourfold excess), considerable thiol–disulfide interchange occurred in our experiments. It is known that ovalbumin undergoes very rapid denaturation in acid urea but slow denaturation in alkaline urea (McKenzie *et al.* 1963). Interchange presumably occurs during unfolding in alkaline urea before the cysteine residues are carboxymethylated. It was found that complete unfolding in acid urea prior to carboxymethylation with a large excess of unlabelled iodoacetic acid kept thiol–disulfide interchange to a minimum.

S1-ovalbumin

The autoradiogram of the separated labelled disulfide-linked half-cystine peptides of S1-ovalbumin is shown in Fig. 2*c*. Thermolytic digestion of this ovalbumin gave an additional peptide C3 which has the sequence Ala-Gln-CMCySO-Gly-Thr-Ser. In some digests another peptide not shown in Fig. 2*c* was formed and had an amino acid composition of $Ile_{0\cdot6}Glu_{2\cdot0}Ala_{1\cdot0}CySO_3H_{0\cdot2}Gly_{1\cdot2}Thr_{1\cdot0}Ser_{1\cdot0}$ and the sequence Ile-Glu-Ala-Gln-CMCySO–Gly-Thr-Ser. The distribution of radioactivity on each peptide sequence A–F is shown in Fig. 4*c*.

Peptides C and D are involved in the disulfide bond and account for 92·9% of the total radioactivity between them, with 3·9% accounted by the remaining four peptides and 3·2% due to miscellaneous radioactive zones.

S2-ovalbumin

The disulfide bond in S2-ovalbumin also occurs between the third and fourth half-cystines from the amino terminal. Separation of the peptides is shown in Fig. 2*d* and the percentage of the total radioactivity on each peptide sequence A–F is shown in Fig. 4*d*. Of the total radioactivity 89·4% resides on the C and D peptides with the remaining radioactivity distributed over the four cysteine-containing peptides (7·3%) and miscellaneous zones (3·3%).

Discussion

The position of the disulfide bond in native ovalbumin has been disputed in the literature. Fothergill and Fothergill (1970) using ^{14}C- and ^{3}H-labelled iodoacetic acid in a double-labelling experiment concluded that the *C*-terminal cysteine in the tryptic peptide sequence Cys-Val-Ser-Pro was involved in the disulfide bond. But this finding was not in accord with the work of Smith (1968) who isolated a large peptide containing the *C*-terminal sequence without disulfide splitting from denatured plakalbumin. The experiments reported here confirm the allocation of the disulfide bond in native ovalbumin by Thompson and Fisher (1978) to the third and fourth half-cystines from the *N*-terminal in the peptide sequences

Phe-Gly-Asp-SerP-Ile-Glu-Ala-Gln-Cys-Gly-Thr-Ser

and

Leu-Gln-Cys.

Thompson and Fisher (1978) identified the disulfide-linked half-cystine peptides of native ovalbumin using a diagonal technique. They also attempted to specifically label the half-cystines with [2-^{14}C]iodoacetic acid; however, their results were equivocal probably due to thiol–disulfide interchange during carboxymethylation resulting in not only the C and D half-cystines being labelled but other cysteines as well. The slow unfolding of ovalbumin in alkaline urea (McKenzie *et al.* 1963) accompanied by a low molar excess of iodoacetic acid over cysteine presumably allows significant thiol–disulfide interchange to occur before carboxymethylation is completed. It is probable that the implication of the *C*-terminal cysteine in a disulfide bond in native ovalbumin by Fothergill and Fothergill (1970), who unfolded and carboxymethylated the cysteine residues of ovalbumin in alkaline urea with a small excess of iodoacetic acid, was an artifact due to thiol–disulfide interchange during carboxymethylation. This problem of thiol–disulfide interchange

is possible with other protein systems particularly if both cystine and cysteine residues are present. The published work on β-lactoglobulin (McKenzie *et al.* 1972) is particularly interesting in that two disulfide forms, 50±5% of each, exist in freshly isolated protein in which the single cysteine occurs in alternate positions at Cys-68 and Cys-70. Unless there was a rapid and complete thiol–disulfide interchange between these residues during carboxymethylation this protein must be synthesized biologically as two alternate structures in equal proportions. McKenzie *et al.* (1972) carboxymethylated using a fourfold excess of [^{14}C]iodoacetamide over cysteine before making 8 M in urea (pH 7·9). Our experiments with ovalbumin suggest that this method and level of blocking reagent may be inadequate to prevent significant thiol–disulfide interchange from occurring in which case this problem could bear reinvestigation. It has been drawn to our attention (H. A. McKenzie, personal communication) that ruminant β-lactoglobulins undergo rapid denaturation in urea over a wide range of pH, unlike ovalbumin which denatures best at low pH. Unpublished results of H. A. McKenzie and D. C. Shaw (1973) using ovalbumin at pH 3·1–3·3 with fourfold excess [^{14}C]iodoacetamide for 16–30 min then pH 8·1 for 15–20 min gave results on subsequent examination consistent with those reported in this paper. In experiments to trap intermediate disulfides in the refolding of reduced proteins Creighton (1974) used 700–1000-fold molar excess of iodoacetic acid over cysteine to block thiol–disulfide interchange.

The ovalbumin conversion is pH-dependent and in the range of ionization of thiol groups which are known to be catalytic in alkaline solutions for thiol–disulfide interchange (Ryle and Sanger 1955). This suggested that the ovalbumin disulfide bond was involved in the formation of ovalbumins of differing heat stability. Smith and Back (1968*b*) investigated the cystine-containing peptides of ovalbumin and S-ovalbumin and found no differences between them but the fractions they analysed were difficult to compare as they were large tryptic peptides containing all the amino acids and not defined amino acid sequences, thereby leaving open the question of thiol–disulfide interchange. In the experiments reported here purified disulfide-linked half-cystine thermolytic peptides of S1- and S2-ovalbumin were isolated and specifically labelled with [2-^{14}C]iodoacetic acid. For both these ovalbumins of differing heat stability the disulfide bond is allocated to the third and fourth half-cystine residues from the *N*-terminal which precludes the involvement of thiol–disulfide interchange in their formation.

Acknowledgments

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