Carboxymethylation of Thiol Groups in Ovalbumin: Implications for Proteins that Contain Both Thiol and Disulfide Groups

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Abstract
The cysteine residues of hen ovalbumin were S-carboxymethylated with non-radioactive iodoacetic acid under various conditions by altering the pH at which the protein was denatured in 8 M urea, by using different molar ratios of non-radioactive iodoacetic acid to cysteine and by varying the time at which carboxymethylation was commenced after denaturing conditions had been applied. Under the various conditions, the thiol groups were carboxymethylated to different extents, the residual thiol groups being measured by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) in the presence of sodium dodecyl sulfate. When ovalbumin is carboxymethylated in alkaline urea, it unfolds slowly and the carboxymethylation is incomplete even with 150-fold excess iodoacetic acid. The known rapid thiol–disulfide exchange that occurs at alkaline pH values makes this method of carboxymethylation unsuitable as a preliminary step for blocking the native cysteine residues of ovalbumin before reduction and labelling the thiol groups formed by reduction of the disulfide bonds. Titration of the thiol groups of ovalbumin in 6 M guanidine hydrochloride or 1% (w/v) sodium dodecyl sulfate at pH 8.2 with 5,5'-dithiobis(2-nitrobenzoic acid) is more rapid than in 8 M urea and these solvents would be preferable for studies of the disulfide-bonded sequences. Denaturation of ovalbumin in acidic 8 M urea is a very rapid process, and under mild acid conditions thiol–disulfide interchange is much slower. Subsequent carboxymethylation of the cysteine residues at alkaline pH with 150-fold excess iodoacetic acid results in complete carboxymethylation and the carboxymethylated ovalbumin can be reduced and labelled with radioactive iodoacetic acid with specific labelling of the half-cystine residues involved in the disulfide bond. The results are discussed in relation to the allocation of half-cystine residues in other protein systems that contain both thiol and disulfide groups.

Introduction
Ovalbumin has recently had its primary and covalent structure elucidated (McReynolds et al. 1978; Thompson and Fisher 1978; Webster and Thompson 1980). It contains one cysteine (linking half-cystine residues 73 and 120) and four cysteine residues (11, 30, 367 and 382). Cystine and cysteine play a significant role in the properties of many proteins, and ovalbumin is one of the readily available proteins that possess both thiol and disulfide bonds.

Huggins et al. (1951) first drew attention to the thiol–disulfide exchange reaction in proteins that contain both thiol and disulfide groups, particularly in urea solutions, but it was Ryle and Sanger (1955) who emphasized the catalytic effects of thiol groups in neutral or alkaline solutions. Thiol–disulfide interchange was shown to be minimal in dilute acid solutions (Ryle and Sanger 1955; Spackman et al. 1960; Cecil 1963). However, under denaturing conditions, even in dilute acid, thiol–disulfide interchange can still be very rapid (Baptist et al. 1976).
It has been noted previously by Thompson and Fisher (1978) and Webster and Thompson (1980) that thiol–disulfide interchange can be a problem in the unequivocal identification of the half-cystine residues of native ovalbumin involved in the disulfide bond and in the allocation of its cysteine residues. The earlier allocation of a particular cysteine-containing peptide sequence as part of the disulfide-linked sequences (Fothergill and Fothergill 1970) was probably a result of incomplete carboxymethylation and disulfide interchange in the alkaline urea solutions used for the carboxymethylations in double-labelling experiments.

The aim of this study was to investigate the carboxymethylation of thiol groups of ovalbumin in 8 M urea solutions under conditions where disulfide interchange is minimal during the denaturation and initial carboxymethylation of the protein. The results are discussed in relation to the allocation of half-cystine residues in other protein systems that contain both thiol and disulfide groups.

Materials and Methods

Preparation of Ovalbumin

Native ovalbumin was prepared from freshly laid hen's eggs by recrystallization as described by Warner (1954).

Preparation of Labelled Disulfide-linked Half-cystine Residues

[2-14C]Iodoacetic acid (54 mCi mmol⁻¹; 1998 MBq mmol⁻¹) was supplied by the Radiochemical Centre, Amersham, England. It was dissolved in water and stored frozen (500 μCi ml⁻¹; 18.5 MBq ml⁻¹).

Native ovalbumin (50 mg) was dissolved in 2.5 ml of distilled and de-ionized water (N.B., all solutions were flushed with nitrogen for at least 20 min before use) and centrifuged in a Sorvall RC2-B centrifuge at 20 000 g for 10 min to remove any denatured protein.

Two methods were used to S-carboxymethylate the cysteine residues before reducing and labelling the disulfide-linked half-cystine residues:

(i) To 0.5 ml of the protein solution 2 ml of 10 M urea–0.2 M Tris-HCl–10 mM EDTA, pH 8.5, was added with stirring. At different periods of time, from 0 to 100 min, either a twofold excess of non-radioactive iodoacetic acid (0.3 mg) or a 150-fold excess (22.3 mg) over cysteine, in 500 μl of 8 M urea–0.2 M Tris-HCl–10 mM EDTA, pH 8.5, was added with rapid mixing, giving final molar concentrations of blocking reagent of 0.54 and 40.3 mM respectively for the 0.067 mM ovalbumin solution.

(ii) To 0.5 ml of the protein solution, 2 ml of 10 M urea–0.2 M HCl–10 mM EDTA, pH approximately 2.5, was added with stirring. Just before making the reaction mixture alkaline, 0.075 mg of non-radioactive iodoacetic acid, dissolved in a minimum of distilled water, was added with stirring. After different periods of time from 30 s to 100 min, 500 μl of 3 M Tris with 0.225 mg of non-radioactive iodoacetic acid (total iodoacetic acid twofold excess over cysteine) titrated to pH 8.5 with 10 M NaOH, was added with rapid mixing (final concentration 0.54 mM), the reaction flask flushed with nitrogen and incubated at 37°C for 20 min in the dark. For a 150-fold excess of non-radioactive iodoacetic acid over cysteine, 5.6 and 16.6 mg (final concentration 40.3 mM) were used, respectively.

Proteins from both methods were recovered following the method of Smith and Back (1968). The pH of the solution was lowered to 4.6 with 17 M acetic acid, 20 ml of water added and the precipitate removed by centrifugation at 15 000 g for 5 min. The precipitate was washed twice and the final precipitate dissolved in 0.5 ml of 0.2 M Tris-HCl–10 mM EDTA, pH 10.0; then 0.16 mg of dithiothreitol was added, the reaction flask flushed with nitrogen and the reduction mixture kept at 37°C for 16 h. The pH was lowered to 8.5 with 6 M HCl, 10 μl of [2-14C]iodoacetic acid added and the reaction flask flushed with nitrogen. After 20 min at 37°C in the dark, alkylation was
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completed with 20 mg of non-radioactive iodoacetic acid and 16 mg of Tris dissolved in 500 \( \mu l \) of 8 M urea–0.2 M Tris-HCl–10 mM EDTA, pH adjusted to 8.5. After a further 20 min at 37°C in the dark, S-carboxymethylated ovalbumin with labelled half-cystine residues was recovered as previously described and the final precipitate digested immediately with thermolysin.

**Enzyme Digestion**

Thermolysin digestion was carried out in 1% (w/v) \( \text{NH}_4\text{HCO}_3 \), pH 8.0, at 37°C for 16 h using 1% enzyme on the weight of substrate. The digest was freeze-dried three times using 10% (v/v) isopropanol as solvent.

**Titration of Thiol Groups in Ovalbumin**

To study the effect of denaturants on the availability of thiol groups, a known weight of ovalbumin (approximately 5 mg) was dissolved in 1 ml of water and diluted with 4 ml of a buffer of pH 8.2 containing 0.2 M Tris–0.02 M EDTA and 10 M urea, or 7.5 M guanidine hydrochloride, or 1.25% (w/v) sodium dodecyl sulfate to give final concentrations of 8 M urea, 6 M guanidine hydrochloride and 1% (w/v) sodium dodecyl sulfate. After 2 min, 20 \( \mu l \) of a 10 mM-methanol solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was added to 1-ml samples of the protein solution and, after mixing, the change in absorbance at 412 nm recorded within 2 min. Reactions were done at room temperature and a sample containing all reagents except the protein was used as a blank. A molar extinction coefficient of 13600 was used (Ellman 1959).

**Measurement of the Degree of Carboxymethylation**

Carboxymethylation of the cysteine residues of native ovalbumin followed the methods previously described except that the protein concentration was determined using \( E_{1 \text{cm}}^{1%} = 7.6 \) (Fasman 1976). After recovery of the protein, the pellet was dissolved in 4 ml of 10 M urea–0.2 M Tris-HCl, pH 8.2. Then 0.5 ml of 3 M Tris-HCl, pH 8.2, was added with 0.5 ml of 5% (w/v) sodium dodecyl sulfate. Analysis of cysteinyl residues followed with the addition of 200 \( \mu l \) of DTNB (0.4% in 0.2 M sodium phosphate buffer, pH 7.0) and measurement of absorbance at 412 nm.

**Peptide Fractionation and Measurement of Radioactivity**

The methods used for peptide fractionation, radioautography and measurement of radioactivity in particular peptides were the same as previously described by Webster and Thompson (1980).

**Results**

**S-carboxymethylcysteine Peptides**

Radioactive peptides containing S-carboxymethylcysteine residues were identified and given the letters A–F to designate the thermolytic peptides in primary sequence from the amino terminal residue (Webster and Thompson 1980). The primary sequences were:

- A Phe-CMCys
- B Phe-Tyr-CMCys-Pro-Ile
- C Phe-Gly-Asp-Ser-Phe-CMCys-Gly-Thr-Ser
- D Leu-Gln-CMCys
- E Leu-Phe-CMCys
- F Phe-Gly-Arg-CMCys.

The predominant form of carboxymethylcysteine was the sulfoxide form, particularly if the freeze-dried enzyme digest was taken up in the pH 6.4 ionophoresis buffer and allowed to stand overnight before fractionation.
Distribution of Radioactivity on the Half-cystine Peptides of Ovalbumin

The distribution of radioactivity on the thermolytic [2-14C]carboxymethylcysteine-containing peptides of ovalbumin isolated from carboxymethylated ovalbumin after reduction of disulfide bonds and reaction with radioactive iodoacetic acid, for the various conditions of pH, the level of blocking reagent, and the time after denaturing conditions had been applied that non-radioactive iodoacetic acid was added, is shown in Fig. 1 for waiting times of 0, 0·5 and 10 min. An effect can be seen in Figs 1a–1d, where the radioactivity on the E peptide (Leu-Phe-Cys) is always characteristically lower than would be expected, and in addition, the radioactivity on the A peptide (Phe-Cys) is characteristically higher than would be expected. Presumably this result may be explained by the partial hydrolysis of the bond between leucine and phenylalanine of the E peptide sequence by thermolysin to give rise to a peptide identical to the A peptide.

Thiol–disulfide Interchange during Carboxymethylation

Thiol–disulfide interchange from the native C–D disulfide-bonded structure cannot be measured in experiments where incomplete carboxymethylation occurs with the unlabelled iodoacetic acid, since subsequent reduction of the disulfide bonds and reaction with radioactive iodoacetic acid will label the previously unreacted thiol groups as well.

McKenzie et al. (1963) reported that alkaline urea only unfolds ovalbumin slowly. This was also shown by experiments to titrate the thiol groups with DTNB in the presence of different denaturants, in which 3·8 moles of thiol groups per mole of ovalbumin were exposed by 6 M guanidine hydrochloride and by 1% (w/v) sodium dodecyl sulfate compared with 0·9 moles by 8 M urea. Whereas both sodium dodecyl sulfate and guanidine hydrochloride unfold the ovalbumin within the time required to measure the thiol groups by the DTNB procedure, the number of thiol groups exposed by 8 M urea continuously increased, although only 2·2 groups were titrated after 2 h.

In the alkaline series of carboxymethylation experiments, an initial rapid disulfide interchange would occur (Ryle and Sanger 1955), the rate of interchange being dependent on the period in 8 M urea at alkaline pH before the iodoacetic acid was added and the molar excess of non-radioactive iodoacetic acid used. With the slow unfolding of ovalbumin in alkaline urea, it appears from the distribution of radioactivity on peptides A and F (Figs 1a and 1b) that the N- and C-terminal cysteine

* The absorbance at 412 nm continues to rise in 8 M urea in contrast to the other denaturants. If left in the urea for 2 h before adding the DTNB reagent, a value of 2·2 was obtained. When the ovalbumin was dissolved in 8 M urea–0·2 M HCl–0·02 M EDTA for 1 min and then brought to pH 8·2 with solid Tris before taking a sample for titration, a stable reading at 412 nm equivalent to 3·3 moles per mole of ovalbumin was obtained.

Fig. 1. Percentage of total radioactivity on the [2-14C]carboxymethylcysteine-containing peptides of S-carboxymethylated ovalbumin after various times and different conditions of denaturation before adding twofold or 150-fold excess iodoacetic acid. Columns A–F designate thermolytic peptide sequences commencing from the amino terminal. (a) Twofold and (b) 150-fold excess of alkaline urea at pH 8·5. (c) Twofold and (d) 150-fold excess of acid urea for the stated time before adjustment to pH 8·5 for carboxymethylation.
residues are either the most likely candidates for initial interchange or, alternatively, the least accessible during the initial carboxymethylation with unlabelled iodoacetic acid. The F sequence is the one claimed by Fothergill and Fothergill (1970) to be involved in the disulfide bond.

A marked reduction in the extent of interchange would be expected in acidic urea, even with low molar excess of iodoacetic acid. It is preferable, however, to limit the period of standing in the acid urea to as little as necessary to ensure complete unfolding. Acid urea denatures ovalbumin very rapidly (McKenzie et al. 1963), exposing all thiol groups. A period of 1 min was found to be sufficient.

Fig. 2. Extent of S-carboxymethylation with non-radioactive iodoacetic acid after various times and different conditions of denaturation before adding twofold or 150-fold excess iodoacetic acid. Unreacted thiol groups were measured using 5,5'-dithiobis(2-nitrobenzoic acid). (a) Twofold and (b) 150-fold excess of alkaline urea at pH 8.5. (c) Twofold and (d) 150-fold excess of acid urea for the stated time before adjustment to pH 8.5 for carboxymethylation.

Estimation of Carboxymethylation

It was found that, due to the slow unfolding of native ovalbumin in alkaline urea, incomplete carboxymethylation of the cysteine residues resulted even after 100 min denaturation in 8 M urea. Initially, there was a sharp increase in the number of cysteine residues carboxymethylated over the first 5 min of denaturation and subsequent carboxymethylation (Figs 2a and 2b) and a slower increase after this time. In addition, the ability to carboxymethylate over this period was dependent upon
the molar excess of non-radioactive iodoacetic acid over cysteine. At zero time, 22% carboxymethylation was achieved for a twofold excess compared with 37.5% for a 150-fold excess. McKenzie et al. (1963) showed that alkylation reagents such as \( p \)-chloromercuribenzoate will increase the rate at which ovalbumin unfolds. A large molar excess of iodoacetic acid increases the rate of denaturation and hence the extent of carboxymethylation per unit of time for the initial part of this experiment. After 100 min preliminary unfolding in 8 M urea, approximately 60% carboxymethylation was achieved.

Even though acid urea denatures ovalbumin very rapidly, exposing all thiols, as can be seen in Fig. 2c, a twofold excess of iodoacetic acid does not carboxymethylate all thiol groups. Approximately 65–72.5% carboxymethylation resulted over the time-course of the experiments. When a fivefold, tenfold and 20-fold excess was used after 5 min denaturation, 93, 97 and 100% carboxymethylation occurred. It is possible that, upon making the reaction mixture alkaline for carboxymethylation, ovalbumin partially refolds, denying the reagent access to buried thiol. The molar excess of non-radioactive iodoacetic acid would determine the rate of carboxymethylation and hence the extent of carboxymethylation before refolding of the molecule. Complete carboxymethylation was achieved, as shown in Fig. 2d, over the entire time-course when a 150-fold excess was used initially.

Thiol–disulfide interchange plays a very significant role in the final distribution of radioactivity on the carboxymethylated half-cystine residues, even though quantitation of this effect is difficult with the ovalbumin system in 8 M urea. It can be seen from Figs 1 and 2 that, even though carboxymethylation increases with time of prior denaturation, further randomization of the disulfide-linked sequences occurs beyond that due to incomplete initial carboxymethylation alone.

Discussion

Thiol–disulfide interchange reactions are of general significance in the behaviour of proteins. This reaction was first discussed at some length by Huggins et al. (1951), who drew attention to the inevitability of the reaction for proteins that contain both thiol and disulfide groups, particularly in urea solutions where rapid gelling occurs due to intermolecular disulfide bond formation. Surprisingly, few proteins contain both thiol and disulfide groups, ovalbumin being one of the most common examples.

The interchange reaction was studied by Ryle and Sanger (1955), who showed the catalytic effects of thiol groups in neutral or alkaline solutions and the inhibition caused by reagents that blocked or destroyed thiol groups. For proteins containing only disulfide groups, trace amounts of thiols probably arise from hydrolytic fission of the disulfide

\[
\text{RSSR} + \text{OH}^- \rightleftharpoons \text{RSOH} + \text{RS}^- .
\]

In strong acid solution, a different mechanism is postulated (Benesch and Benesch 1958) with \( \text{RS}^+ \) prompting the interchange and, as Ryle and Sanger (1955) found, thiols inhibit the interchange, presumably by promoting the back reaction in the equation

\[
\text{RSSR} + \text{H}^+ \rightleftharpoons \text{RS}^+ + \text{RSH} .
\]
The reaction is minimal in dilute acid solutions in the pH range 1–6.5 (Sanger 1953; Spackman et al. 1960; Cecil 1963), and in isolating disulfide-bridged peptide fragments, the most suitable enzyme has been pepsin (Spackman et al. 1960; Brown and Hartley 1966) with its pH optimum in the 2–4 region.

The addition of reagents such as N-ethylmaleimide and iodoacetic acid, which remove traces of thiol, inhibits the interchange at neutral and alkaline pH. In their work on the disulfide bridges of ribonuclease, however, Ryle and Anfinsen (1957) found no difference in the pattern of peptides produced by subtilisin at pH 8 in the presence of N-ethylmaleimide, yet they obtained incorrect pairing of two disulfide bridges, suggesting that disulfide interchange was possibly a problem in their experiments.

When significant concentrations of thiol are present, as in the present experiments with ovalbumin, the situation is more complex because there will be competition between the disulfide interchange reaction

\[ R^1 - S - S - R^2 + R^3 S^- \rightleftharpoons R^1 - S - S - R^3 + R^2 S^- \]

and the removal of thiol, e.g.

\[ RS^- + I^- + CH_2COO^- \rightarrow RSCH_2COO^- + I^- , \]

both reactions being promoted by increasing pH (Eldjarn and Pihl 1957). Because of this competition, the accessibility of thiol groups as well as the relative excess of blocking reagent will influence the rate of interchange before blocking of thiol groups is complete, as demonstrated in the results of the present study. It should also be noted that Creighton (1974) used a 700–1000-fold molar excess of iodoacetic acid over cysteine in experiments, to prevent thiol–disulfide interchange in the refolding of reduced pancreatic trypsin inhibitor.

Even at low pH values, thiol–disulfide interchange reactions can occur very rapidly. With human haemoglobin or native globin in which the two reactive β-chain cysteine residues had been coupled with DTNB to create disulfide bonds, followed by attempts to isolate DTNB-treated globin by dropping into acetone-HCl (0·15 M HCl or less) at −20°C, the result was a mixture of the disulfide-linked products with interchange and oxidation of almost all the uncoupled ‘masked’ thiol groups (Baptist et al. 1976). Such loss or rearrangement accompanies the denaturation process in acetone-HCl at −20°C, but it does not occur if irreversible blocking groups introduced by N-ethylmaleimide or iodoacetic acid are used.

In the determination of disulfide-bridged sequences in proteins containing both thiol and disulfide residues, there is a problem in trying to specifically label the thiol groups, e.g. with [2-14C]iodoacetic acid, without disulfide interchange, since the high cost of the radioactively labelled reagent limits the molar excess that can be used. It is obviously preferable to block the thiols using a large molar excess of unlabelled reagent and subsequently reduce and label the half-cystine residues with labelled reagent.

In their work on ovalbumin, Fothergill and Fothergill (1970) used C[14]- and H[3]-labelled iodoacetic acid (no indication of molar excess was given) in an attempt to elucidate the tryptic peptides involved in the disulfide bond. They implicated the C-terminal F peptide sequence Cys-Val-Ser-Pro. It was shown by Thompson and Fisher (1978) and Webster and Thompson (1980), however, that this sequence
is not involved in the disulfide bond. Double-labelling experiments of the type described by Fothergill and Fothergill (1970) with ovalbumin are almost certain to give misleading results because of either disulfide interchange during the initial carboxymethylation in alkaline urea solution or in the case of ovalbumin an incomplete initial carboxymethylation due to the slow unfolding of the protein molecules to expose the thiol groups, or both.

The extent of interchange in ovalbumin is dependent upon the rate of denaturation of the protein at a particular pH, hence its accessibility to the thiol-blocking reagent, the time of denaturation before carboxymethylation and the molar excess of blocking reagent over cysteine. It was found in this study that denaturation in acidic urea for 60 s, then carboxymethylation of the cysteine residues at alkaline pH with a large excess of blocking reagent, followed by subsequent reduction and labelling with [2-14C]iodoacetic acid gave very specific labelling of the native half-cystine residues with complete carboxymethylation and minimal thiol–disulfide interchange. Although reaction of excess iodoacetic acid with side chain groups of lysine, histidine or methionine is possible (Gundlach et al. 1959), with the approach recommended in this paper the side reactions would occur with unlabelled iodoacetic acid rather than with the radioactive labelled reagent which need not be used in large excess. Consequently, such side reactions should not complicate the isolation and characterization of radioactively labelled peptides.

In the present work, we have studied the carboxymethylation of ovalbumin in 8 M urea because Fothergill and Fothergill (1970) used urea in their disulfide allocation experiments. Moreover, there has been a comprehensive study of the denaturation of ovalbumin in urea solutions (McKenzie et al. 1963; Tanford 1968). It would be preferable to use guanidine hydrochloride or sodium dodecyl sulfate as denaturant rather than urea because the thiol groups of ovalbumin are obviously exposed more rapidly in these solvents. It has also been found with other proteins that guanidine hydrochloride solutions are more effective than urea solutions in exposing thiol groups that are not exposed in the native protein. For example, the subunit of β-glucuronidase revealed 0·4 residues of cysteine in 8 M urea compared with two residues in 6 M guanidine hydrochloride or sodium dodecyl sulfate (Hawley 1973).

Hirs (1967a) recommended the use of 8 M urea as solvent for the reduction of disulfide bonds because of ‘difficulties encountered in the purification of commercially available guanidine salts’ and also (Hirs 1967b) drew attention to the adverse effect of EDTA and guanidine hydrochloride on the stability of cystine peptides, even though it was uncertain whether the deleterious effects observed by Spackman et al. (1960) were due to these substances themselves or to impurities in the reagents used. Methods for the purification of guanidine hydrochloride are now available (Nozaki 1972) as it is the solvent of choice for producing random coils from proteins (Tanford 1968), and has more recently been recommended (Konigsberg 1972) for the reduction of disulfide bonds in proteins.

There are some proteins in which disulfide interchange has apparently not occurred to a significant extent during the initial carboxymethylation of cysteine residues in the presence of an intramolecular disulfide bond. For example, the thiol groups in lipoamide dehydrogenase (dihydrolipoamide reductase NAD+) (Brown and Perham 1972, 1974) were specifically S-carboxymethylated by the gradual addition of guanidine hydrochloride to 6 M in the presence of iodoacetic acid, but no indication of the molar excess of blocking reagent was given.
It should be pointed out that disulfide interchange is only a problem where both cystine and cysteine are present. A reduced protein can readily be labelled with limited amounts of radioactive iodoacetic acid, even in the presence of reducing-agent thiol, since thiol groups of the protein are more reactive than those of the reagent (Thompson and O'Donnell 1961; Crestfield et al. 1963) and high specific activities are obtainable.

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References


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