Selective reduction of a disulphide bridge in hen ovotransferrin

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Brief treatment of iron-saturated hen ovotransferrin with dithiothreitol selectively cleaves the disulphide bridge between residues 478 and 671, which is in the C-terminal domain of the protein. The reduced alkylated protein is less stable than the native protein, and its iron-binding properties are different. A fluorescent derivative was prepared by coupling N-iodoacetyl-N'- (5-sulpho-1-naphthyl)ethylenediamine to the thiol groups.

Transferrins are extracellular iron-binding proteins which possess a bilobar structure in which the homologous N-terminal and C-terminal halves of the chain fold up into separate globular domains, each with an iron-binding site. Disulphide bridges occur within each domain, though none pass between them. In hen transferrin there are six bridges in the N-domain and nine in the C-domain. Of the C-terminal bridges, six are homologous to the N-terminal bridges and the remaining three have no N-terminal counterparts (Fig. 1). It is possible that the ability of urea/polyacrylamide-gel electrophoresis to separate the N-terminal and C-terminal monoferric forms of transferrin (Makey & Seal, 1976) depends on the presence of different numbers of bridges in each domain (Williams et al., 1982).

Thornton (1981) has noted that in extracellular globular proteins there is a marked tendency for disulphide bridges to be local, i.e. to join points less

Fig. 1. A string model of hen ovotransferrin showing the positions of disulphide bridges (Williams et al., 1982)
than 45 residues apart in the sequence. In hen transferrin five of the six bridges common to the two domains are local, although bridge number 3 (Fig. 1) joins points about 80 residues apart. The three extra C-terminal bridges are all long-range, connecting points which are from 193 to 275 residues apart. It has been suggested that these bridges are phylogenetically more recent acquisitions than the common six (Williams, 1982).

Thornton (1981) also pointed out that most disulphide bridges are buried in the protein structure. In a few cases they are exposed at the surface, where they can be reduced without prior denaturation of the protein. Here we show that selective cleavage of one disulphide bridge in the C-terminal domain of the iron complex of hen ovotransferrin is readily effected, with a consequent decrease in the stability of the protein.

Materials and methods

Materials

Ovotransferrin was isolated from hen's- and duck's-egg white by the method of Williams (1968). Iron was removed by the method of Warner & Weber (1951). Activated thiol-Sepharose 4B was obtained from Pharmacia. Dithiothreitol was obtained from Park Scientific Ltd., Northampton, U.K. I-AEDANS, 2-Py-S-S-2-Py and Nbs2 were obtained from Sigma Chemical Co.

Methods

Preparation of half-molecules. N-Terminal and C-terminal half-molecules of ovotransferrin were prepared either by trypsin digestion of partially iron-saturated samples of the protein (Williams, 1974, 1975) or by a modification of the method of Brock et al. (1976). In the latter method, iron-saturated ovotransferrin was dissolved in buffer (0.05 M-Tris/HCl/0.02 M-CaCl2, pH 7.9) and digested with trypsin (enzyme/substrate, 1:50, w/w) for 4 h at 37°C. The digest was then subjected to chromatography on DEAE-Sepharose with a linear buffer gradient of 0.05–0.5 M-Tris/HCl, pH 7.9. By this method the N-terminal and C-terminal half-molecules were obtained in a homogeneous state, as judged by SDS/polyacrylamide-gel electrophoresis.

Reduction and alkylation. Protein samples were dissolved in 0.1 M-Tris/HCl, pH 7.9, at a concentration of 2% (w/v). The solution was cooled in ice and dithiothreitol was added (dithiothreitol/protein, 1:20, w/w). After 20 min either sodium iodoacetate or I-AEDANS was added in 3-fold molar ratio to the amount of dithiothreitol used. After 10 min the mixture was applied to a column (20 cm × 2 cm) of Sephadex G-25 in 0.01 M-NH4HCO3 and the protein peak was freeze-dried. The alkylated protein was separated from unmodified material by chromatography on DEAE-Sepharose with a linear gradient of 0.1–0.5 M-Tris/HCl, pH 7.9. Protein peaks were dialysed and freeze-dried. The purity of the modified proteins was examined by starch-gel electrophoresis and SDS/polyacrylamide-gel electrophoresis.

Isolation of cysteine peptides. Covalent chromatography, based on the method of Svenson et al. (1977), was used to identify the cysteine residues produced by reduction of iron-saturated hen ovotransferrin with dithiothreitol. Reduction was carried out as described above and was terminated by applying the solution to a column (20 cm × 2 cm) of Sephadex G-25 equilibrated with 1 mM-2-Py-S-S-2-Py in a mixture of formic acid and acetic acid (both 0.2 M). The protein peak was collected and the excess 2-Py-S-S-2-Py was removed by passage through a second column of Sephadex G-25 in formic acid/acetic acid. Pepsin was then added to the protein peak (enzyme/substrate, 1:50, w/w) and digestion was carried out for 15 h at 37°C. The peptide-containing solution was passed through a column (5 cm × 1 cm) of thiol-Sepharose in formic acid/acetic acid. Unbound peptides were washed off with the acid mixture, and cysteine peptides were then eluted with 50 mM-β-mercaptoethanol in 0.1 M-ammonium acetate, pH 8.5. The eluted peptides were freeze-dried and purified by paper electrophoresis at pH 6.5, 3.5 or 2.1 (Ambler, 1963). Electrophoretic mobilities of peptides at pH 6.5 relative to that of aspartic acid were measured (Offord, 1966).

Thiol assays. Thiol contents of reduced protein samples were determined by several methods. In one method the reduced protein was passed through a column (20 cm × 2 cm) of Sephadex G-25 in 0.01 M-HCl. The protein peak was collected and thiol assay was performed with Nbs2 (Ellman, 1959). Alternatively, the A280 of AEDANS-protein was measured against a blank of unmodified protein, assuming a molar absorption coefficient for the 5-sulphonaphthyl group of 3400. Finally, alkylated protein samples were hydrolysed with 6 M-HCl at 105°C for 24 h, and CM-cysteine was measured with an amino acid analyser.

Urea denaturation. The ability of increasing concentrations of urea to denature the reduced and alkylated protein was studied by two methods (Evans & Williams, 1980). In the first, iron-free protein samples were dissolved in solutions containing different concentrations of urea, and their ability to take up iron from a saturating dose of Fe(NO3)2 was estimated by the A470. In the second, iron-free samples were subjected to electrophoresis in polyacrylamide gels containing a gradient of urea concentration normal to the direction of electrophoresis (Creighton, 1979).
Iron-binding properties. The distribution of iron between the N-terminal and C-terminal binding sites during the progressive saturation of native and modified ovotransferrins with iron was studied by electrophoresis in polyacrylamide gels containing 6M-urea (Makey & Seal, 1976). The loss of iron from saturated protein samples dissolved in 0.1M-sodium citrate, pH5.65, was followed as described by Williams (1975).

Sequential methods. N-Terminal residues of peptides were determined by the dansyl method (Gray & Hartley, 1963). Automatic sequencing was carried out with an Anachem Solid-Phase Peptide Sequencer.

Electrophoretic methods. Starch-gel electrophoresis was carried out by the discontinuous method of Poulak (1957). SDS/polyacrylamide-gel electrophoresis was performed as described by Evans & Williams (1978).

Results

Reduction and alkylation

Starch-gel electrophoresis showed that, after reduction of iron-saturated hen ovotransferrin or the iron-saturated C-terminal half-molecule followed by alkylation with I-AEDANS, a fast-moving fluorescent component was present, in addition to small amounts of the unchanged original protein. These components were completely separated from the unchanged materials by chromatography on DEAE-Sepharose. On the other hand, neither the N-terminal half-molecule of hen ovotransferrin nor intact duck ovotransferrin gave any new products under these conditions. Alkylation with iodoacetate gave a similar but non-fluorescent derivative of hen ovotransferrin.

Thiol assay

In one experiment iron-saturated hen ovotransferrin was reduced with 5% dithiothreitol for different times. The thiol contents are shown in Table 1. This result suggests that there is rapid reduction of one disulphide bridge, followed by much slower reduction of other bridges. Spectrometry of the purified AEDANS derivative of the C-terminal half-molecule prepared after reduction for 20 min gave 2.3 mol of 5-sulphonaphthyl group/mol of protein. Similarly amino acid analysis of the AEDANS derivatives of hen ovotransferrin and of the C-terminal half-molecule and of the carboxymethyl (CM) derivative of hen ovotransferrin gave 1.9–2.1 mol of CM-cysteine/mol of protein in each case.

Identification of cysteine residues

After a reduction of iron-saturated hen ovotransferrin with 5% dithiothreitol for 20 min, pepsin digestion, covalent chromatography and subsequent performic acid oxidation gave only two peptides on paper electrophoresis at pH6.5.

Peptide 1. Mobility relative to aspartic acid was +0.04 and the amino acid composition was CySO3H1.0, Asp1.7, Thr1.7, Gly1.0, Ile0.7, His0.7, -Arg1.0, Glucosamine was also present. The N-terminal residue was isoleucine. By referring these data to the amino acid sequence of ovotransferrin (Jeltsch & Chambon, 1982), it is likely that peptide 1 corresponds to the glycopeptide sequence Ile-His-Asn(CH0)-Arg-Thr-Gly-Thr-CySO3H-Asn, where (CHO) represents the glycan.

Peptide 2. Mobility relative to aspartic acid was −0.24 and the amino acid composition was CySO3H1.1, Asp2.0, Thr1.0, Ser1.0, Pro1.0, Lys1.1. The N-terminal residue was lysine, and automatic solid-phase sequencing gave the structure (−)-Thr-CySO3H-Asn-Pro-Ser (the first residue was lost in this procedure). The N-terminal residue was not obtained in the solid-phase sequencing experiment. Peptide 2 thus corresponds to the sequence Lys-Thr-CySO3H-Asn-Pro-Ser-Asp.

The cysteic acid residues in these two peptides occupy positions 478 and 671 in the amino acid sequence, and in the native protein they are joined to form disulphide bridge number 7 (Williams et al., 1982) in the C-terminal half of the molecule. Reduction for longer periods of time or the use of larger amounts of dithiothreitol resulted in some additional weak bands appearing in the electrophoretogram, confirming the result given in Table 1. These bands were not studied.

Urea denaturation

An experiment to estimate the ability of apo-ovotransferrin and apo-CM-ovotransferrin to bind iron in the presence of urea is shown in Fig. 2. The modified protein begins to lose its iron-binding capacity at a lower urea concentration than is necessary for the native protein. Urea-gradient/polyacrylamide-gel electrophoresis also showed that denaturation of the CM-protein occurred at a lower urea concentration than for the native

Table 1. Thiol content of hen ovotransferrin reduced with 5% dithiothreitol at 0°C for different times

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<tr>
<th>Time (h)</th>
<th>Thiol content (mol/mol of protein)</th>
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<tr>
<td>0.5</td>
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<td>2</td>
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<td>4</td>
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<td>7</td>
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<td>23</td>
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protein. In both cases the denaturation appears to occur in a single step. The same result was obtained with carboxamido-ovotransferrin, in which the reduced protein was alkylated with iodoacetamide (Fig. 3).

Iron-binding properties

Titration of iron-free protein samples with FeN(Ac)₃ showed no significant difference between the iron-binding capacities of native ovotransferrin and CM-ovotransferrin (Fig. 4). However, at subsaturating iron concentrations, less iron was bound to the C-terminal site of the modified protein. Urea/polyacrylamide-gel electrophoresis showed virtual absence of the C-terminal monoferric complex during the course of iron addition. This was confirmed by trypsin digestion of 30% iron-saturated AEDANS-ovotransferrin, when Sephadex G-100 gel filtration of the digest showed that only high-Mᵣ product was a fragment which was identified as the N-terminal half-molecule. When the native protein was subjected to the same treatment, three high-Mᵣ products were obtained: diferric ovotransferrin and both the N-terminal and C-terminal half molecules (J. Williams & K. Moreton, unpublished work). Williams et al. (1978) have shown by

Fig. 2. Iron-binding capacity of native ovotransferrin (●) and CM-ovotransferrin (○) in urea solutions
Saturating amounts of iron were added to the iron-free protein containing different concentrations of urea. For experimental details, see Evans & Williams (1980).

Fig. 3. Electrophoresis of a mixture of apo-ovotransferrin (a) and apo-carboxamidomethyl-ovotransferrin (b) on a urea-gradient/polyacrylamide gel
For experimental details, see Evans & Williams (1980).

Fig. 4. Titration of iron-free ovotransferrin (●) and iron-free CM-ovotransferrin (○) with FeN(Ac)₃
Iron uptake was measured by A₄₅₀. Each 5μl addition of 0.01M-FeN(Ac)₃ corresponds to 0.266 mol of Fe/mol of protein. In each case saturation occurs at 1.9 mol of Fe/mol of protein.
Double-isotope labelling that iron in subsaturating doses binds to both domains of the native protein, although in unequal amounts.

The loss of iron from fully saturated ovotransferrin and CM-ovotransferrin in acid solution is shown in Fig. 5. The modified protein loses iron more rapidly than does the native protein.

**Immunological properties**

CM-ovotransferrin and native ovotransferrin gave a precipitin reaction which indicated immunological identity when tested with rabbit anti-ovotransferrin serum in the Ouchterlony test (Williams, 1975).

**Discussion**

Treatment of iron-saturated hen ovotransferrin with 5% dithiothreitol for 20 min at 0°C results in the specific cleavage of the disulphide bridge between residues 478 and 671 (bridge number 7 in Fig. 1). The same selectivity appears to operate when the isolated half-molecules are used instead of the whole protein, although in this case we have not identified the cysteine residues involved. The two cysteine residues thus produced are available for further modification, such as the coupling of two fluorescent AEDANS groups, as we have shown. By contrast, iron-saturated duck ovotransferrin appears to possess no exposed disulphide bridges.

The overall conformation of the modified protein is not grossly altered, as judged by its immunological properties, and it retains the normal iron-binding capacity. On the other hand, iron-binding to the C-terminal domain is weaker than normal and the stability of the protein to urea denaturation is decreased. There is, as present, no direct evidence that the modified protein would be less stable than normal under conditions in vivo. This is nonetheless likely to be the case, and it would explain the existence of this bridge (Williams, 1982).

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**References**


