The identification of protected tyrosine residues in iron-ovotransferrin

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Tetranitromethane reacts with essentially all 21 tyrosine residues of iron-free ovotransferrin. In iron-ovotransferrin, 7 mol of tyrosine/mol of protein are unreactive. Peptides containing the unreactive tyrosine residues were isolated from digests of nitrated iron-ovotransferrin. By comparing the structures of the peptides with the amino acid sequence of ovotransferrin it is found that there are ten protected residues occupying positions 42, 82, 92, 188, 191, 319, 415, 431, 521 and 524 in the polypeptide chain. The problem of identifying the tyrosine residues that form bonds with the metal atoms is discussed.

The involvement of tyrosine residues in iron binding by transferrin was first indicated by the observation that six protons are released from side groups with pKₐ values greater than 10.0 when two atoms of iron are bound (Warner & Weber, 1953). Later it was found that six fewer tyrosine residues can be titrated in iron-ovotransferrin than in the iron-free protein (Wishnia et al., 1961). Supporting evidence for the role of tyrosine was provided by resonance Raman spectroscopy (Tomimatsu et al., 1976; Carey & Young, 1974), proton magnetic resonance (p.m.r.) (Woodworth et al., 1970), u.v. difference spectroscopy (Tan & Woodworth, 1969), circular dichroism (c.d.) (Mazurier et al., 1976) and electron-paramagnetic-resonance (e.p.r.) spectroscopy (Aasa et al., 1963; Windle et al., 1963). Most of these studies show that three tyrosine residues are involved in the binding of each ferric ion, although Tan & Woodworth (1969) obtained a value of two.

Chemical modification of tyrosine by nitration with tetranitromethane (Bekkorovainy & Grohlich, 1972; Line et al., 1967; Teuwissen et al., 1973; Tsao et al., 1974), iodination (Phillips & Azari, 1972) or acetylation (Komatsu & Feeney, 1967) destroys the iron-binding ability of iron-free transferrin. In the iron-saturated protein, 2–3 mol of tyrosine/metal atom bound remain unmodified and the modification of the other tyrosine residues does not lead to loss of the bound iron. These reagents do not distinguish between tyrosine residues in the iron-binding sites of iron-free transferrin and those elsewhere in the molecule. With periodate, however, Azari & Phillips (1970) found that for three to five tyrosine residues out of the total number of 21 in iron-free ovotransferrin underwent rapid oxidation with loss of the iron-binding ability. No destruction of tyrosine occurred on periodate treatment of the iron-protein. Geoghegan et al. (1980) confirmed this observation and proposed that the oxidizable tyrosine residues are those that function as ligands to the metal atoms. They also found that urea denaturation largely protects the binding-site tyrosine residues against oxidation, which suggests that their special reactivity may depend on the integrity of the native structure of the protein.

An essential step in elucidating the structure of the iron-binding sites is the location of the tyrosine ligands in the primary structure of the protein. The only study in which this has been attempted appears to be that by Tsao et al. (1974), in which peptides containing unmodified tyrosine were detected in trypsin digests of nitrated iron-ovotransferrin. Attempts to purify these peptides were not successful and the work was greatly hampered by the fact that the amino acid sequence of ovotransferrin was not known at that time, Jeltsch & Chambon (1981) have recently deduced the amino acid sequence of ovotransferrin from the nucleotide sequence of the corresponding mRNA. This sequence contains 21 tyrosine residues and when the N-terminal and C-terminal halves of the sequence are aligned so as to give the greatest number of identical matching residues, it is seen that there are six pairs of matching tyrosine residues (Williams et al., 1981). These ‘conserved’ pairs of tyrosine residues are at positions 82 and 415, 92 and 431, 188 and 521, 191 and 524, 239 and 581 and at 325 and 662. In each pair the first-quoted residue is in the N-terminal domain and the second is in the C-terminal domain of the protein. Since the iron-binding properties of the two domains are very similar, although not identical (Aisen & Listowsky, 1980), it would be
expected that the tyrosine ligands occupy conserved positions.

The experiments described in the present paper, which were carried out in 1968, attempt to identify those tyrosine residues in the iron-ovotransferrin complex that are unreactive towards tetranirotromethane. Ten such residues are identified and some of these form conserved pairs. No straightforward identification of the iron ligands can be made from these results.

Materials and methods

Hen ovotransferrin was prepared as described by Williams (1968). Iron was removed by the method of Warner & Weber (1951) and saturation with iron was carried out by adding iron(III) nitrilotriacetate to a 1% (w/v) solution of ovotransferrin in 0.1M NaHCO₃/0.1M-Tris/HCl, pH 8.0, until the $A_{465}$ indicated saturation.

Treatment with tetranirotromethane

Tetranirotromethane (Aldrich Chemical Co. Inc., Milwaukee, WI, U.S.A.) was used as a 10% (v/v) solution in 95% (v/v) ethanol. (Caution: tetranirotromethane is a skin and lung irritant and is highly explosive in the presence of impurities.) This was added to a 1% (w/v) solution of iron-free ovotransferrin or iron-saturated ovotransferrin in 0.1M NaHCO₃/0.1M-Tris/HCl, pH 8.0, to give a 10-fold excess of the reagent over the tyrosine content of the protein. After 3 days at room temperature the protein was separated from low-molecular-weight reaction products and reagents by passage through a column (50 cm x 2 cm) of Sephadex G-25 in 0.01M NH₄HCO₃ and freeze-dried.

Determination of tyrosine content

Proteins and peptides were hydrolysed with 5.7M-HCl in sealed evacuated tubes at 105°C for 24 h. Amino acids were determined with a Technicon amino acid analyser. Nitrotyrosine was also estimated by dissolving nitrated protein samples in 0.1M-NaHCO₃/0.1M-Tris/HCl, pH 8.0, and measuring the $A_{430}$. A value of 4100 litre·mol⁻¹·cm⁻¹ was assumed for the molar absorption coefficient of 3-nitrotyrosine. In the case of the nitrated iron-saturated protein, iron was removed before measuring the $A_{430}$.

Enzyme digests

Nitrated protein samples were dissolved in water to give 2% (w/v) solutions. The pH was adjusted to 2.0 with HCl. Pepsin (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) was added to give an enzyme-to-substrate ratio of 1:40 (w/w) and incubation at 37°C was carried out for 7 h. A further lot of pepsin was added and incubation was continued overnight. The digest was dried by rotary evaporation. In one experiment, nitrated iron-ovotransferrin was reduced and alkylated by the method of Crestfield et al. (1963) and digested with trypsin (Worthington), by using an enzyme-to-substrate ratio of 1:50 (w/w). The buffer was 1% (NH₄)₂CO₃ and digestion was continued for 12 h at 37°C.

Fractionation of peptides

Preliminary fractionation of peptides was carried out by ion-exchange chromatography on a column (150 cm x 2 cm) of Dowex 50 X2 resin (Bio-Rad AG, 50W, X2, 200–400 mesh) with volatile pyridine/ acetic acid buffers (Schroeder, 1967). The column was loaded with 2 g of protein digest in 20 ml of pyridine/acetic acid buffer (pH 2.7, pyridine concentration 0.2 m). Peptides were eluted with a linear gradient from pH 3.1 (pyridine concn. 0.2 m) to pH 5.0 (pyridine concn. 2.0 m). A 2-litre portion of each buffer was used in forming the gradient. The column was surrounded by a water jacket at 37°C. A flow rate of 45 ml/h was used and 9 ml fractions were collected.

Fractions containing tyrosyl or tryptophyl peptides were detected with the Folin reagent (Lowry et al., 1951). A sample (0.5 ml) of each fraction was mixed with 3.5 ml of 2% (w/v) Na₂CO₃ in 0.5M NaOH and 0.2 ml of Folin-Ciocalteu reagent (diluted 1:1 with water). The $A_{500}$ was measured after 30 min. Peptide-containing fractions were pooled and rotary-evaporated. The selectivity of this reagent for tyrosyl and tryptophyl peptides was achieved by the omission of the copper reagent.

Further purification of peptides was carried out by high-voltage paper electrophoresis at pH 6.5, 3.5 and 2.1 (Ambler, 1963). Papers were stained with a cadmium acetate/ninhydrin reagent (Heilmann et al., 1957) and with 1-nitroso-2-naphthol (Jepson & Smith, 1953), which gives a red colour with tyrosine-containing peptides but which does not react with 3-nitrotyrosine. Electrophoretic mobilities of peptides at pH 6.5 relative to that of aspartic acid were measured (Offord, 1966).

Amino-acid-sequence determination

The amino acid compositions of peptides were determined with a Technicon TSM-1 analyser after hydrolysis with 5.7M-HCl at 105°C for 24 h. Sequences were determined by the dansyl–Edman method (Gray & Hartley, 1963: Gray, 1967).

Results

Tyrosine determinations

The nitrotyrosine content of tetranirotromethane-treated iron-free ovotransferrin, as estimated from the $A_{320}$, was 19.9 ± 0.6 mol/mol of protein. For tetranirotromethane-treated iron-ovotransferrin the
nitrotyrosine content from the $A_{430}$ was 12.3 ± 0.5 mol/mol of protein. By using the amino acid analyser the following tyrosine contents were determined: 22.2 mol/mol of untreated ovotransferrin, 7.1 ± 0.7 mol/mol of nitrated iron-ovotransferrin and 0.9 mol/mol of nitrated iron-free ovotransferrin.

Tyrosine-containing peptides

Thirteen tyrosine-containing peptides were obtained from pepsin digests of nitrated iron-ovotransferrin. The electrophoretic mobilities are expressed relative to aspartic acid, for which $m = -1$.

Peptide P1 ($m = 0$). The amino acid composition was Ser$_{0.7}$, Gly$_{1.0}$, Ala$_{1.0}$, Tyr$_{1.0}$. The sequence was Tyr-Ser-Gly-Ala.

Peptide P2 ($m = -0.31$). The amino acid composition was Asp$_{0.9}$, Ser$_{2.0}$, Gly$_{1.2}$, Gly$_{0.9}$, Ala$_{1.6}$, Tyr$_{1.0}$, Arg$_{1.3}$. The N-terminal sequence Asp-Glu-Arg-Pro was determined. It is likely that this peptide is contained within the sequence Thr-Asp-Glu-Arg-Pro-Ala-Ser-Tyr, which was obtained by Elleman & Williams (1970) as their peptide P$_{106}$. The excess of serine and glycine in peptide P2 is probably due to contamination.

Peptide P3 ($m = -0.66$). The amino acid composition was Asp$_{1.0}$, Gly$_{2.3}$, Ala$_{0.3}$, Tyr$_{0.5}$, Arg$_{1.1}$. The sequence was Ala-Glu-Arg-Tyr-Asp-Asp-Glu.

Peptide P4 ($m = 0$). The amino acid composition was Thr$_{0.6}$, Gly$_{2.0}$, Ala$_{1.0}$, Phe$_{0.6}$, Tyr$_{1.0}$. The partial structure Phe-Gly-Tyr-Thr-(Gly, Ala) was determined.

Peptide P5 ($m = 0$). The amino acid composition was Thr$_{0.6}$, Gly$_{1.0}$, Ala$_{1.0}$, Tyr$_{1.0}$. The sequence was Tyr-Thr-Gly-Ala.

Peptide P6 ($m = -0.21$). The amino acid composition was Thr$_{2.5}$, Ser$_{1.0}$, Gly$_{1.1}$, His$_{0.5}$, Tyr$_{1.0}$. The partial structure His-Thr-Glu-(Gly, Ser$_{2}$, Thr$_{3}$, Tyr) was determined and it corresponds to the peptide His-Thr-Glu-Gly-Ser-Thr-Thr-Ser-Tyr, whose structure was fully determined by T. C. Elleman (unpublished work).

Peptide P7 ($m = +0.5$). The amino acid composition was Gly$_{1.2}$, Tyr$_{0.5}$, Lys$_{1.3}$, His$_{1.0}$. The sequence was His-Glx-Lys-Tyr. From the mobility the net charge at pH 6.5 must be greater than 1. The sequence is therefore His-Glx-Lys-Tyr.

Peptide P8 ($m = 0$). The amino acid composition was Gly$_{1.0}$, Leu$_{1.1}$, Tyr$_{1.0}$ and the sequence was Tyr-Leu-Gly.

Peptide P9 ($m = -0.43$). The amino acid composition was Val$_{0.6}$, Gly$_{1.0}$, Tyr$_{1.0}$ and the sequence was Val-Tyr-Glu.

Peptide P10 ($m = -0.29$). The amino acid composition was Asp$_{1.1}$, Thr$_{1.0}$, Ser$_{1.0}$, Gly$_{1.0}$, Pro$_{1.3}$, Ala$_{1.1}$, Tyr$_{1.0}$, Arg$_{1.0}$. The partial sequence Thr-Asp-Glu-Arg-Pro-Ala-(Ser, Tyr) was determined.

Peptide P11. This peptide differed from peptide P10 only in the possession of C-terminal phenylalanine. Treatment with carboxypeptidase A released phenylalanine, tyrosine and serine as shown by paper electrophoresis at pH 2.1.

Peptide P12. The amino acid composition was Gly$_{1.1}$, Tyr$_{1.7}$, Phe$_{1.0}$ and the sequence was Tyr-Phe-Gly-Tyr.

Peptide P13 ($m = 0$). The amino acid composition was Asp$_{1.0}$, Pro$_{0.8}$, Ala$_{1.0}$, Tyr$_{0.6}$. The mobility indicated the presence of asparagine, and the partial structure Asn-Ala-(Pro, Tyr) was obtained.

Four tyrosine-containing peptides were obtained from a trypsin digest of nitrated iron-ovotransferrin.

Peptide P14. The amino acid composition was Asp$_{2.0}$, CMCys$_{1.0}$, Ser$_{1.5}$, Glu$_{2.1}$, Tyr$_{0.7}$, Lys$_{0.8}$. The partial structure Tyr-Asp-Asp-Glu-Ser-(Glx, CMCys, Ser, Lys) was determined. (CMCys represents carboxymethylcysteine.)

Peptide P15. The amino acid composition was Pro$_{1.5}$, Gly$_{1.0}$, Ala$_{1.0}$, Tyr$_{1.0}$ and the sequence was Tyr-Ala-Arg-Pro-Gly.

Peptide P16 ($m = 0$). The amino acid composition was Asp$_{1.0}$, Ser$_{1.0}$, Pro$_{1.0}$, Gly$_{1.2}$, Ala$_{1.0}$, Tyr$_{1.3}$. The partial structure Asn-Ala-Pro-Tyr-Gly-(Tyr, Ser) was determined.

Peptide P17. The amino acid composition was CMCys$_{0.5}$, Asp$_{1.1}$, Thr$_{0.9}$, Ala$_{1.1}$, Ile$_{0.8}$, Leu$_{1.2}$, Tyr$_{1.0}$, Lys$_{1.0}$. The amino acid sequence was Ala-Thr-Tyr-Leu-Asp-CMCys-Ile-Lys.

Discussion

Ovotransferrin contains 21 tyrosine residues (Jeltsch & Chambon, 1981). Tetranitromethane treatment of iron-free ovotransferrin gives essentially complete nitration of tyrosine, and the small amount of remaining tyrosine has not been studied further in these experiments. The amount of unreactive tyrosine in iron-ovotransferrin is about 7 mol/mol of protein (7.6 by the $A_{430}$ and 7.1 by amino acid analysis). These results are in complete agreement with those of Tsao et al. (1974).

Fig. 1 shows the amino acid sequence of hen ovotransferrin as deduced from the mRNA sequence (Jeltsch & Chambon, 1981). Of the 17 peptides containing unmodified tyrosine residues, 15 can readily be located in this sequence. In the case of peptide P7, the structure differs from that deduced from the mRNA in that glutamine replaces the glutamic acid at position 519. At present, nine sites in ovotransferrin appear to show polymorphism (Williams et al., 1981). Peptide P9 (Val-Tyr-Glu) cannot be definitely identified in the sequence, but it may be tentatively suggested that it corresponds to Ile-Tyr-Glu at positions 81–83. Peptide T2 cannot be fitted to the sequence and is presumably a contaminant. From these data (Fig. 1) the tyrosine residues that are protected from reaction with
tetranitromethane in iron-ovotransferrin are those at positions 42, 82 (tentative), 92, 188, 191, 319, 415, 431, 521 and 524.

Since the binding of two iron atoms to ovotransferrin protects 7 mol of tyrosine/mol of protein and ten protected tyrosine residues have been identified, it follows that for at least some of these residues the protection is only partial. Those tyrosine residues which are iron ligands will probably be completely protected, since nitration of the iron complex does not cause loss of iron. In the present experiments the extent of the protection conferred on each tyrosine residue could not be estimated because of the non-quantitative recovery of peptides during the many purification steps.

In addition to the protection of tyrosine residues that are directly bound to the metal atoms, other tyrosine residues could be made unavailable to the reagent by the conformational changes that accompany iron binding. From the present results no distinction between the two types of protected tyrosine residues can be made. However, some effort at distinguishing the two types can be made on the assumption that the iron ligands in the N-terminal and C-terminal domains of transferrin are homologous. Thus eight of the protected tyrosine residues occur as 'conserved' pairs at positions 82, 92, 188 and 191 in the N-terminal domain and at positions 415, 431, 521 and 524 in the C-terminal domain. There was no evidence of protection of the other two pairs of 'conserved' tyrosine residues (at positions 239, 325, 581 and 662). The protected tyrosine residues at positions 42 and 319 in the N-terminal domain have no matching tyrosine partners in the C-terminal domain and it is therefore unlikely that they are iron ligands. Thus it is now...
possible to restrict the choice of the six iron-binding tyrosine residues to eight out of the total number of 21. Clearly, it would be of great interest to know the identities of the four tyrosine residues that undergo selective oxidation by periodate (Geoghegan et al., 1980).

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References