The cross-linking of tyrosine residues in apo-ovotransferrin by treatment with periodate anions

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The iron-binding ability of apotransferrins is rapidly abolished in the reaction with periodate anions, which destroys 4 mol of tyrosine per mol of protein. Treatment of ovotransferrin with cyanogen bromide and tryptic digestion of the glycopeptide fragment demonstrated the existence of an intramolecular cross-link in the C-terminal domain of the oxidized protein. The cross-linked residues were identified as Tyr-421 and Tyr-524 and the product is similar in structure to 3,3'-dityrosine.

INTRODUCTION

Periodate anions (IO_4^-) are unreactive towards simple alcohols but are able to oxidize diols when in 1,2- or certain 1,3-configurations (reviewed by Dryhurst, 1970). Such reactions, termed Malapradian oxidations, proceed only because the anion is able to co-ordinate both functional groups to form a cyclic diester intermediate. Monohydric phenols are generally resistant to oxidation (Clamp & Hough, 1965), whereas catechols and dihydroquinones are readily oxidized (Windrath, 1956), but the mechanism(s) of these reactions is unknown. Tashiro (1963, 1966) has shown that in the treatment of tyrosine with periodate 3,3'-dityrosine and its derivatives are major oxidation products. The reactions between proteins and periodate have not been extensively studied: *N*-terminal serine and threonine behave as Malapradian substrates by means of their 1-amino-2-hydroxy groups, as in the rapid oxidation of corticotropin (Dixon, 1962). Of the remaining common amino acid residues, methionine, cysteine, cystine and still more slowly tyrosine, tryptophan and histidine are also oxidized by periodate (Chatterjee *et al.*, 1961; Clamp & Hough, 1965; Atassi, 1967; Rippa *et al.*, 1981).

The mammalian transferrins are glycoproteins with M_r values of approx. 80000 that are able to bind an Fe^{3+} ion and an anion reversibly in each of their two homologous domains. Ovotransferrin is found only in avian egg-whites, where it is free of iron and functions as a bacteriostatic agent.

Azari & Phillips (1970) found that the iron-binding ability of hen apo-ovotransferrin is rapidly destroyed upon treatment with periodate, but the diferric protein is resistant to oxidation. Amino acid analysis showed the loss of about 4 mol of tyrosine/mol of protein. Geoghegan *et al.* (1980) subsequently confirmed the oxidation of tyrosine, but no loss of tryptophan was reported. In addition, this reaction appears to be inhibited by denaturation in concentrated urea solutions (Geoghegan *et al.*, 1980). Oxidation of methionine residues of transferrin by periodate was not detected until later, but this does not contribute to the loss of iron-binding ability (Yamasaki *et al.*, 1982). Finally, Penner *et al.* (1983) showed that these reactions are common to hen

ovotransferrin, human serotransferrin and human lactotransferrin.

Little attempt has been made to understand the mechanism of the reaction between periodate and transferrins, but Geoghegan *et al.* (1980) have suggested that the former behaves as an affinity reagent, which is attracted to the iron-binding sites of transferrins by the basic residues thought to reside at or near these regions (reviewed by Chasteen, 1983). More direct evidence supporting this mechanism has been gained from kinetic studies of the oxidation of apo-ovotransferrin (Hsuan, 1986), but it remains to show precisely which tyrosine residues are modified and whether or not electrostatic attraction alone is sufficient to explain the postulated selective oxidation of residues involved in binding iron.

Tyr-521 and Tyr-524 in the C-terminal domain of ovotransferrin have been implicated as probable iron-binding residues by previous studies (reviewed by Chasteen, 1983); accordingly, a fragment containing these residues was isolated following CNBr treatment of the periodate-oxidized derivative by employing the single glycan on Asp-473 as a marker. Subsequent tryptic digestion showed that periodate had cross-linked Tyr-524 to Tyr-431, both of which are thought to be located at the iron-binding site (Chasteen, 1983). Several physical properties of this oxidation product and 3,3'-dityrosine are compared.

EXPERIMENTAL

Materials

Horseradish peroxidase (EC 1.11.1.7), dansyl chloride and dansyl-amino acids were from BDH Chemicals, Poole, Dorset, U.K.; bovine pancreatic trypsin (diphenylcarbamoyl chloride-treated; type XI) (EC 3.4.21.4) and CNBr were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Hen egg-white ovotransferrin was prepared by the method of Williams (1968) and made free of iron as described by Evans & Williams (1978).

Methods

Preparation of oxidized apo-ovotransferrin. To a solution of apo-ovotransferrin (2 mg/ml) in 0.05 M-Hepes/

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NaOH buffer, pH 7.4, 0.1 M-NaIO₄ was added to give a concentration of 5 mM at room temperature and in the dark, with stirring continuously. After 25 min a 15-fold molar excess of 10% (v/v) ethylene glycol over periodate was added to quench the reaction, and the solution was dialysed before being freeze-dried.

Treatment with CNBr. The method described by Kingston & Williams (1975) was used to treat both unoxidized and oxidized apo-ovotransferrin with CNBr and to isolate the major glycopeptides following reduction and alkylation. Final purification of the required glycopeptide fragment of the unoxidized protein was achieved by chromatography on a Sephadex G-50 column as described by Kingston & Williams (1975), but that from the oxidized protein was purified on a Sephadex G-75 column (40 cm × 1.6 cm) equilibrated with 5% (v/v) acetic acid. The carbohydrate-containing fractions were detected by the orcinol/H₂SO₄ method of Winzler (1955).

Treatment of fragments with dimethyl sulphoxide/CNBr reagents. The method of Huang *et al.* (1983) was used to cleave the oxidized glycopeptide selectively at tryptophan residues with CNBr following treatment with the acetic acid/HCl/dimethyl sulphoxide oxidizing mixture. The latter reaction was carried out at 4 °C for 2 h and the former at room temperature for 17 h in the dark. The final mixture was degassed, and formic acid was added (final concn. 40%, v/v) to dissolve the precipitate before fractionation on a Sephadex G-50 column (98 cm × 1.5 cm) previously equilibrated with 5% (v/v) formic acid.

High-voltage paper electrophoresis. Peptides were separated by electrophoresis at pH 6.5 or 2.1 (Ambler, 1963) and stained with cadmium acetate/ninhydrin (Heilman *et al.*, 1957) or phenanthraquinone (Yamada & Itano, 1966) reagents. Electrophoretic mobilities were measured relative to that of aspartic acid ($m_r = -1.00$) at pH 6.5 and serine ($m_r = 1.00$) at pH 2.1 in accordance with Offord (1966).

H.p.l.c. peptide mapping. The glycopeptides (2 mg/ml) in 0.5 M-NH₄HCO₃, pH 8.0, were digested with 2% (w/w) trypsin at 37 °C for 4 h; the reaction was quenched by immersion of the mixture in boiling water before it was freeze-dried. The products were resuspended in buffer A [0.1% (v/v) trifluoroacetic acid] and applied to a reverse-phase h.p.l.c. column (Lichrosorb 10 RP18) (25 cm × 0.46 cm) previously equilibrated with 5% (v/v) buffer B [0.1% (v/v) trifluoroacetic acid in acetonitrile] in buffer A. Peptides were eluted with a staggered gradient of 5–60% (v/v) buffer B in buffer A (Fig. 4) with a constant flow rate of 1 ml/min.

End-group determination. N-Terminal dansyl-amino acids were prepared by the method of Gray (1967), and the products were analysed by two-dimensional polyamide t.l.c. as described by Hartley (1970).

Preparation of 3,3'-dityrosine. This was based on the work of Williams & Lowe (1971). The pH of a 2.5 mM-tyrosine solution in 1% (w/v) NH₄HCO₃ was adjusted to 9.2 with 33% (w/v) NH₃. Then 10% (v/v) tetranitromethane in 95% (v/v) ethanol was added to give a 1.5-

fold molar excess over tyrosine. After 3 h at room temperature the mixture was acidified with 2 M-HCl and dried by rotary evaporation at 37 °C. The residue was dissolved in water and fractionated by high-voltage paper electrophoresis at pH 2.1 (60 V/cm for 75 min). After exposure to NH₃ fumes a band, strongly fluorescent (blue) under long-wave u.v. light and positive to the cadmium acetate/ninhydrin reagent, was eluted with water. Purification was by reverse-phase h.p.l.c. [Lichrosorb 10 RP18 column (25 cm × 0.46 cm)] with a linear gradient of 5–40% (v/v) buffer B in buffer A. Purity was assessed by automated amino acid analysis.

Isolation of oxidized tyrosine residues. The peptide containing the oxidized residues was hydrolysed in 6 M-HCl/0.1% (w/v) phenol *in vacuo* for 16 h at 110 °C. The mixture was dried *in vacuo* over solid NaOH and then fractionated by reverse-phase h.p.l.c. as described above for the purification of 3,3'-dityrosine.

Fluorescence spectroscopy. Samples were dissolved in 0.25 M-NH₄HCO₃, pH 9.0; relative fluorescence excitation (emission wavelength 410 nm) and emission (excitation wavelength 316 nm) spectra were measured by using a Perkin-Elmer 3000 fluorescence spectrometer.

Mass spectrometry. Unmodified material dissolved in dichloromethane was introduced on the solid probe of a Finnigan 4000 quadrupole mass spectrometer. Spectra were recorded by using the INCOS 2300 data system, while the temperature was controlled manually.

Amino acid analysis. Hydrolyses were performed in 6 M-HCl/0.1% (w/v) phenol *in vacuo* overnight at 110 °C. The hydrolysates were dried *in vacuo* over solid NaOH and suspended in 0.025 M-HCl for application to a Rank-Hilger Chromaspek J180 automated amino acid analyser.

Polyacrylamide-gel electrophoresis. This was carried out as described by Fairbanks *et al.* (1971) in the presence of 0.1% (w/v) SDS.

RESULTS

Chemical proteolyses

The gel-filtration elution profiles of the fragments produced by CNBr treatment of unoxidized and oxidized apo-ovotransferrin are shown in Fig. 1. The pooled material from peaks B and C, or oxB and oxC, was reduced and carboxymethylated before fractionation, again by gel filtration on Sephadex G-100 (Fig. 2). The profiles of the unoxidized protein are as expected from the work of Kingston & Williams (1975), but the profiles of the oxidized protein clearly differ from these. Carbohydrate was found primarily in peaks BCd and oxBc, but the latter was eluted earlier than BCd (Fig. 2).

The fractionation of peptides derived from fraction oxBc by chemical proteolysis on the C-terminal side of tryptophan residues is shown in Fig. 3. High-voltage paper electrophoresis at pH 6.5 separated both peaks III and IV into four bands, as shown by the cadmium acetate/ninhydrin reagent; the compositions of these bands are shown in Table 1.

Fig. 1. Elution profile of ovotransferrin fragments

Following treatment with CNBr unoxidized (a) and oxidized (b) apo-ovotransferrin were fractionated by Sephadex G-100 gel-filtration chromatography in 5% (v/v) formic acid as described in the text. The peaks in (a) are labelled in accordance with Kingston & Williams (1975).

Fig. 2. Elution profile of BC and oxBC fractions

Fractions corresponding to peaks B + C and to oxB + oxC (Fig. 1) were pooled before reduction and alkylation. Fractionation was by Sephadex G-100 gel-filtration chromatography in 5% (v/v) formic acid as described in the text. The unoxidized protein peaks (a) are labelled in accordance with Kingston & Williams (1975).

M_r of fragment oxBCc

The M_r value of fragment BCd, estimated by Kingston & Williams (1975) by using SDS/polyacrylamide-gel electrophoresis, varied from 11700 to 17800. By using the same technique the M_r values of fragments BCd and oxBCc are estimated here to be 15500 and 22500 respectively. The M_r of fragment BCd, calculated from its primary structure, is 11990 (Kingston & Williams, 1975).

Fig. 3. Elution profile of oxBCc fragments

Fragment OxBCc was cleaved at tryptophan residues and the products were fractionated by Sephadex G-50 gel-filtration chromatography in 5% (v/v) formic acid as described in the text.

Table 1. Peptides derived from fragment oxBCc by chemical proteolysis at tryptophan residues

Peaks III and IV (Fig. 3) were fractionated by high-voltage paper electrophoresis at pH 6.5. Peptides were detected by using the cadmium/ninhydrin reagent and eluted for amino acid analysis. Compositions are given as molar proportions relative to one amino acid (*) in each peptide. The sequence positions were readily determined by comparison of the composition with the primary structure of ovotransferrin (Fig. 7). Abbreviation: CmCys, carboxy-methylcysteine; Hse, homoserine; HseL, homoserine lactone.

Peptide	Amino acid composition	Position
(III)a	CmCys (+), Asx (1.54), Thr (1.63), Ser (1.02), Glu (0.37), Gly (0.95), Ala (0.32), Val (1.22), Leu (0.53), His (1.09), Lys (3.00)*, Arg (1.54)	446-464
(III)b	CmCys (+), Asx (1.64), Thr (2.88), Ser (0.98), Gly (3.85), Ala (2.69), Val (1.06), Leu (0.51), His (0.91), Lys (3.00)*, Arg (1.53)	446-464
(III)c	CmCys (+), Asp (1.57), Thr (2.42), Ser (1.47), Hse (0.90), Glu (0.43), Pro (1.61), Gly (2.85), Ala (1.59), Val (1.16), Met (0.73), Ile (0.84), Leu (0.40), His (1.00)*, Lys (1.96), Arg (1.34)	446-468
(III)d	CmCys (+), Asp (1.76), Thr (3.14), Ser (0.17), Hse (1.11), Glu (0.46), Pro (1.41), Gly (1.94), Ala (1.88), Val (1.04), Met (0.58), Ile (1.13), Leu (0.14), His (0.27), Lys (0.29), Arg (1.00)*	446-468
(IV)e	Asp (1.05), Ser (0.08), Hse (1.72), Glu (1.64), Ala (0.77), Leu (1.02), His (0.15), Lys (1.00)*	558-563
(IV)f	Ser (0.18), Hse (1.80), Glu (0.08), Pro (2.90), Val (0.44), Ile (1.00)*, HseL (+), His (0.07)	465-468
(IV)g	Asp (1.49), Hse (1.63), Glu (1.11), Pro (0.55), Ala (1.37), Ile (0.20), Leu (1.04), HseL (+), His (0.05), Lys (1.00)*	558-563
(IV)h	Hse (1.86), Pro (1.70), Val (0.66), Ile (1.00)*, HseL (+)	465-468

Enzymic proteolyses

H.p.l.c. mapping of tryptic digests of fragments BCd and oxBCc (Fig. 4) produced three and eight major peaks at 276 nm respectively; the amino acid composition

Fig. 4. H.p.l.c. maps of digests of peptides BCd and oxBCc

Tryptic digests of peptides BCd (a) and oxBCc (b) were dissolved in buffer A (200 μ l, 0.5 mg/ml) and separated by reverse-phase h.p.l.c. at a constant flow rate of 1 ml/min as described in the text.

of each band resolved by high-voltage paper electrophoresis of these peaks is shown in Table 2.

Properties of 3,3'-dityrosine

The standard 3,3'-dityrosine had an electrophoretic mobility at pH 2.1 of 0.87 relative to serine and 1.13 relative to tyrosine, showing close agreement with the results obtained by Fry (1984). Purification by reverse-phase h.p.l.c. gave two major peaks of retention time 20.8 and 25.3 min; the former was identified as 3,3'-dityrosine by its fluorescence spectra (Fig. 5), and automated amino acid analysis of this material gave a single peak that was eluted at the position of histidine.

Characterization of oxidized tyrosine residues

Comparison of the composition of peptide oxH (Table 2) with the primary structure of ovotransferrin suggested that it contained two peptides that are linked by modified tyrosine residues. Fractionation of the acid hydrolysate of this peptide by reverse-phase h.p.l.c. gave two strong peaks at 276 nm of retention time 16 min (DT.I) and 23 min (DT.II) (Fig. 6). Automated amino acid analysis and reverse-phase h.p.l.c. of a tyrosine standard (retention time 16 min) showed DT.I to be tyrosine. DT.II gave two peaks on amino acid analysis of height ratio approx. 1:1.2 at the positions of phenylalanine and histidine respectively. The fluorescence spectra of the hydrolysate of peptide oxH (Fig. 5) show clear differences to those of 3,3'-dityrosine: the emission maximum occurs at a longer wavelength, suggesting greater resonance stabilization of the oxH product compared with 3,3'-dityrosine.

In addition to the similar properties on amino acid analysis, the mass spectra of 3,3'-dityrosine and DT.II are extremely similar (Table 3), but the weak molecular ion at m/z 354 lies below the M_r value of 3,3'-dityrosine (360).

N-Terminal analysis of peptide oxH showed dansyl-threonine and bisdansyl-tyrosine derivatives; the fluorescence of the latter was more intense, as expected from its structure and the partial destruction of the former during acid hydrolysis (Gray, 1967).

Table 2. Amino acid compositions of BCd and oxBCc tryptic peptides

The products of the tryptic digestion of peptides BCd and oxBCc were separated by reverse-phase h.p.l.c. (Fig. 4); each peak was fractionated by high-voltage paper electrophoresis at pH 6.5 before amino acid analysis. Compositions and positions are given as described in Table 1. Abbreviations; Cho, carbohydrate. CmCys, carboxymethylcysteine.

Peptide	Amino acid composition	Position
oxA	CmCys (0.56), Asx (2.16), Ser (2.12), Glx (2.37), Tyr (1.11) Lys (1.00)*	415–423
oxB1	Ala (2.40), Val (1.57), Arg (1.00)*	433–438
oxB2	CmCys (0.47), Thr (1.03), Ser (0.95), Gly (1.42), Ala (1.22), Val (1.00), His (0.97), Arg (1.00)*	453–460
oxB3	Asx (1.23), Thr (1.69), Ser (1.17), Glx (2.61), Gly (2.22), Val (1.20), Ile (0.77), His (0.92), Lys (1.00)*	540–552
oxC1	Ala (2.35), Val (1.51), Arg (1.00)*	433–438
oxC2	Asx (0.58), Gly (1.12), Ile (0.43), Leu (0.59), His (0.80), Arg (1.00)* Cho	469–474
oxC3	Asx (1.70), Ala (3.12), Lys (1.00)*	555–559
oxD1	Asx (1.76), Leu (1.33), Lys (1.00)*	446–449
oxD2	Asx (1.90), Ala (1.58), Lys (1.00)*	555–559
oxD3	CmCys (0.46), Glx (1.27), Val (1.09), Leu (1.06), Lys (1.00)*	530–534
oxE	Asx (3.07), Ser (1.21), Gly (1.95), Ala (1.86), Val (1.17), Leu (1.07), Lys (1.00)*	†
oxF1	Asx (1.32), Lys (1.00)*	553–554
oxF2	CmCys (0.78), Ser (1.36), Glx (2.58), Pro (2.00), Gly (3.53), Ile (1.00), Leu (1.92), Lys (1.00)*	498–512
oxF3	Asx (1.10), Gly (1.52), Ala (1.36), Val (1.00)*, Phe (0.99)	535–539
oxG	CmCys (0.31), Asx (3.11), Thr (1.61), Ser (2.41), Glx (1.69), Pro (2.99), Gly (3.11), Ala (1.41), Tyr (0.75), Phe (1.52), Arg (1.00)*	475–497
oxH	Asx (1.18), Thr (1.89), Ser (1.30), Glx (1.33), Pro (1.40), Gly (2.35), Ala (2.34), Leu (1.00)*, Tyr (0.93), Phe (1.40), His (1.29), Arg (2.12)	424–432 +
D	Asx (0.76), Ala (1.39), Lys (1.00)*	555–559
I	Gly (1.68), Tyr (1.89), Phe (1.00)*	521–524
G	CmCys (1.91), Asx (2.74), Thr (1.69), Ser (2.65), Glx (2.47), Pro (3.67), Gly (2.16), Ala (0.48), Tyr (0.98), Phe (1.53), Arg (1.00)*	475–496

† No position assigned.

DISCUSSION

The glycopeptide fragment BCd, produced by CNBr treatment of ovotransferrin, is known to comprise residues 469–563 (Kingston & Williams, 1975; Williams *et al.*, 1982). The corresponding fragment oxBCc is larger than fragment BCd, as shown by SDS/polyacrylamide-gel electrophoresis and by gel-filtration chromatography.

Fig. 5. Fluorescence spectra of dityrosines

Emission spectra (i) were recorded with an excitation wavelength of 316 nm and excitation spectra (ii) were recorded with an emission wavelength of 410 nm. Samples were dissolved in 0.25 M-NH₄HCO₃, pH 9.0. (a) 3,3'-Dityrosine at a concentration of approx. 3.2 nmol/ml; (b) DT.II at a concentration of approx. 1.5 nmol/ml; (c) buffer. Concentrations were estimated from automated amino acid analysis assuming a 2-fold greater colour yield with ninhydrin at 570 nm relative to tyrosine.

Fig. 6. Fractionation of hydrolysate of peptide oxH

The acid hydrolysate of peptide oxH in buffer A (200 μ l, 500 nmol/ml) was fractionated by reverse-phase h.p.l.c. as described in the text at a constant flow rate of 1 ml/min.

Table 3. Major peaks of the mass spectra of dityrosines

Spectra were recorded of unmodified samples of 3,3'-dityrosine and DT.II (Fig. 6). The peaks at m/z 149 (*) are due to phthalic anhydride produced by the spectrometer.

m/z	
3,3-Dityrosine	DT.II
57	58
74	
87	86
101	101
111	111
129	129
142	142
149*	149*
167	167
185	185
199	199
213	213
241	241
245	245
255	255
259	259
267	267
273	
279	279
287	
298	298
354	354

This may be due firstly to the cross-linking of residues leading to a two-chain peptide and secondly to the inhibition of CNBr action by the oxidation of methionine residues on treatment with periodate (Yamasaki *et al.*, 1982). The situation is clarified by the results of chemical proteolysis at tryptophan residues: the positions of tryptic peptides (III)a to (IV)h (Fig. 7) show that CNBr had cut at methionine residues 411, 468 and 563 and that the fragments 411-468 and 469-563 form a two-chain peptide, oxBCc (calculated M_r 6350 + 11990). Use of less stringent oxidation conditions than those described by Geoghegan *et al.* (1980), Yamasaki *et al.* (1982) and Penner *et al.* (1983) still results in the complete abolition of iron-binding ability (Hsuan, 1986) and may decrease the number of methionine residues that are reported to be oxidized by the reaction conditions of previous workers (Yamasaki *et al.*, 1982; Penner *et al.*, 1983).

The positions of tryptic peptides from fragments BCd and oxBCc (Fig. 7) confirm the identity of these glycopeptide fragments. Peptide oxH is of particular interest as it appeared to have lost two tyrosine residues and to contain two peptides that could not be separated by electrophoresis; the absence of any unmodified peptides from these positions in the protein suggests that peptide oxH contains two of the four oxidized residues of the whole protein. The strong affinity of peptide oxH for the stationary phase of the h.p.l.c. column is consistent with its five aromatic groups and the results of the dansylation of peptide oxH imply that tyrosine residues 431 and 524 are cross-linked.

The cross-linked tyrosine residues are identical with 3,3'-dityrosine in retention time on automated amino acid analysis and consistent with the results reported by

Fig. 7. Partial primary structure of ovotransferrin

The sequence shows points of CNBr action at methionine (↓) and tryptophan (↑) residues near the glycosylated asparagine residue (473). The positions of peptides derived from peaks III and IV (Fig. 3) and of tryptic peptides from peaks BCd and oxBCc (Fig. 2) are also shown.

Amado *et al.* (1984) for 3,3'-dityrosine. Comparison of the mass spectra confirms the structural similarity of these compounds. The significance of the small number of different peaks in the mass spectra, the different retention times on reverse-phase h.p.l.c. and the dissimilar fluorescence spectra is not clear, but suggests that peptide oxH contains tyrosine residues that are linked in a different manner to a 3,3'-bond.

The cross-linking of phenols occurs via a free-radical mechanism when reagents such as tetranitromethane, which was employed for the synthesis of 3,3'-dityrosine, are used. Insulin and trypsin can be treated with this reagent (Williams & Lowe, 1971) or with a H_2O_2 /peroxidase mixture (Amado *et al.*, 1984) to yield 3,3'-dityrosine. Apo-ovotransferrin, however, gives no 3,3'-dityrosine with tetranitromethane, although all the tyrosine residues are modified by this reagent (Williams & Lowe, 1971). This suggests, first, that the oxidation of apo-ovotransferrin by periodate does not follow the free-radical mechanism of tetranitromethane, secondly, that the product of the periodate reaction may therefore be expected to differ from 3,3'-dityrosine, and, finally, that the role of charge is not critical in these reactions as both the nitronium cation (NO_2^+) and the periodate anion readily attack the tyrosine residues at the iron-binding sites.

The mechanism of the periodate oxidation of ovotransferrin is not known, but as an alternative to a free-radical mechanism it is possible that periodate forms a Malapradian, cyclic, intermediate complex with two tyrosine

residues, similar to that suggested by Kaiser & Weidman (1965) for the oxidation of catechols by periodate. The specificity of the ovotransferrin reaction would therefore be a consequence of the stereochemistry of the tyrosine ligands, possibly enhanced by the ability of proximal basic residues to bind complex anions (Hsuan, 1986). In this way the periodate anion can be termed an affinity reagent for the iron-binding sites of transferrins, and preliminary results from limited chemical and enzymic proteolysis experiments suggest that the corresponding *N*-terminal-domain tyrosine residues of apo-ovotransferrin (92 and 191) are also cross-linked by periodate oxidation (J. J. Hsuan, unpublished work).

Finally, from the above discussion the pair of tyrosine residues that react with periodate anions may also act as iron ligands, particularly as these are two of three tyrosine residues that are conserved in both domains of ovo-, sero-, lacto- and melano-transferrins (Rose *et al.*, 1986).

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