On the Structure of Ovotransferrin. III. Nitration of Iron–Ovotransferrin and Distribution of Tyrosines Involved in Iron-Binding Activity†

Dean Tsao, Parviz Azari,* and Jerry L. Phillips

ABSTRACT: Nitration of iron-free ovotransferrin with tetranitromethane resulted in the disappearance of all 20 tyrosine residues with a complete loss of iron-binding activity. Under the same conditions, however, iron-saturated ovotransferrin showed the disappearance of 13–14 tyrosines with complete retention of iron-binding activity. The modified tyrosines were not quantitatively recovered as 3-nitrotyrosines. Identification of polypeptide segments containing the six–seven unmodified tyrosine residues in nitrated iron–ovotransferrin was approached by employing tryptic hydrolysis and CNBr cleavage of the modified protein. Ion-exchange chromatography of the tryptic hydrolysate of nitrated iron–ovotransferrin indicated that the number of tyrosine-containing peptides was significantly reduced as compared to that of native ovotransferrin. CNBr cleavage of the modified iron–ovotransferrin produced four homogeneous fragments. The unmodified tyrosines were found only in the two largest fragments, designated NO_2CF1- and NO_2CF2, which have been previously assigned to the N- and C-terminal ends of the native protein, respectively. It is suggested that the two iron-binding sites are located in these fragments.

Ovotransferrin (also known as conalbumin), a metal-binding protein from hen's egg white, binds 2 mol of metal, most notably ferric iron, per mol of protein (mol wt 76,700) (Feeney and Komatsu, 1966). Presently, it is believed that the two metal-binding sites are noninteracting and nonequivalent (Aasa, 1972), although it has been assumed that the amino acid side chains of the protein which are responsible for metal-binding activity are similar for both sites (Feeney and Komatsu, 1966). Although the precise chemical nature of the metal-binding sites is as yet unknown, various chemical and physical studies have implicated both tyrosine and histidine residues in the chelation of iron. In particular, spectral studies of both ovotransferrin and the related human serum transferrin have demonstrated two–three tyrosines per each binding site (Wishnia *et al.*, 1961; Aasa *et al.*, 1963; Tan and Woodworth, 1969; Woodworth *et al.*, 1970). Similar results have been obtained by chemical modification, employing acetylation and iodination of ovotransferrin (Komatsu and Feeney, 1967; Phillips and Azari, 1972), nitration of human serum transferrin (Line *et al.*, 1967), and nitration of human lactoferrin (Teuwissen *et al.*, 1973).

In the present study, an attempt was made to devise a technique which would allow the identification of those tyrosine residues involved in the iron-binding activity of ovotransferrin. This work was based on the hypothesis that the binding of iron by ovotransferrin would specifically protect from chemical modification those tyrosines at the iron-binding sites (Azari and Feeney, 1961; Line *et al.*, 1967; Teuwissen *et al.*, 1973). To this end tyrosine residues in Fe–ovotransferrin[1] have been modified with tetranitromethane, a reagent essentially specific for tyrosine. A product was obtained which showed no loss of chromogenic activity, was approximately 90% homogeneous by acrylamide gel electrophoresis, and which still contained six–seven unmodified tyrosines. Subsequent cleavage of this nitrated Fe–ovotransferrin[1] with CNBr and fractionation of the resulting fragments revealed free tyrosines in only two fragments, NO_2CF1 and NO_2CF2, containing 5 and 4 mol of free tyrosine/mol, respectively. Hence, the data indicate that the combination of nitration and CNBr cleavage constitutes a workable method for identifying those tyrosine residues involved in the iron-binding activity of ovotransferrin. Furthermore, these data substantiate the observation that NO_2CF1 contains one of the iron-binding sites of ovotransferrin (Tsao *et al.*, 1974b). It is also suggested that the second iron-binding sites of ovotransferrin may be contained, at least in part, in NO_2CF2.

Experimental Procedure

Materials. Ovotransferrin was a 4× crystallized preparation, as described previously (Azari and Baugh, 1967). The protein appeared homogeneous by sedimentation velocity and by acrylamide gel electrophoresis. Cyanogen bromide was purchased from Eastman Kodak (Organic Chemical Division). Propionic acid (buffer grade) was a product of Pierce Chemical Co. Tetranitromethane was obtained from Aldrich Laboratories. Aminex AG-1-X2 (minus 400 mesh) and Bio-Gel P-150 (100–200 mesh) were purchased from Bio-Rad Laboratories, and Dowex 1-X4 was from J. T. Baker Chemical Co. L-1-Tosylamido-2-phenylethyl chloromethyl ketone treated trypsin was obtained from Worthington. All other reagents were of the highest quality available and used without further purification.

Methods. Fe–ovotransferrin was prepared by the addition of sufficient iron citrate to a solution of ovotransferrin in 0.05 M Tris buffer (pH 8.2) to form a saturated complex (the iron

† From the Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80521. *Received August 10, 1973.* This investigation was supported by Research Grant GB 30956 from the National Science Foundation. The material was taken in part from a thesis submitted by D. T. to the Graduate College of Colorado State University in partial fulfillment for the degree of Doctor of Philosophy in Biochemistry.

[1] Abbreviations used are: Fe–ovotransferrin, saturated iron complex of ovotransferrin, containing 2 mol of Fe(III)/mol of protein; NO_2, indicates nitrated protein; RCM, reduced and carboxymethylated.

added provided a 2.5× molar excess of Fe(III) over ovotransferrin). The mixture was incubated at room temperature for 6 hr prior to the addition of ethanol and tetranitromethane.

Nitration was conducted essentially as described by Sokolovksy *et al.* (1966). The reaction was performed at room temperature in 0.05 M Tris–10% ethanol (pH 8.2). The protein, 40 mg of ovotransferrin or Fe–ovotransferrin, was dissolved in 4 ml of buffer. An aliquot of 100 μl of diluted tetranitromethane was added to provide an 8.4× molar excess of reagent over tyrosine content (ovotransferrin contains 20 tyrosine residues per molecule). The reaction was allowed to proceed for 18 hr. To remove excess reagent and by-products, the reaction mixture was passed through a short column of Sephadex G-25 equilibrated with 0.1 M NH_4HCO_3 (pH 8.5). The effluent containing the nitrated protein was collected and freeze-dried. The extent of 3-nitrotyrosine production was determined spectrophotometrically from the increase in absorbance at 428 nm, using an extinction coefficient of 4.1×10^3 M^{-1} cm^{-1} for 3-nitrotyrosine. Amino acid analysis, after acid hydrolysis, was also used to quantitate tyrosine modification. Tryptophan was determined by a colorimetric procedure employing *p*-dimethylaminobenzaldehyde (Phillips and Azari, 1971).

Removal of protein-bound iron by Dowex 1-X4 ion-exchange chromatography and reduction–carboxymethylation of ovotransferrin and modified ovotransferrin were performed as described previously (Tsao *et al.*, 1974b).

Tryptic hydrolysis of reduced and carboxymethylated protein was conducted as follows. All samples were suspended in distilled water to a final concentration of 1%. The pH was adjusted to 8.1 with 0.1 N NaOH. L-1-Tosylamido-2-phenylethyl chloromethyl ketone trypsin, in an amount equal to 2% of the protein by weight, was added to each solution. The incubation temperature was 37°, and the pH was maintained at 8.1 by adding 0.1 N NaOH with a microburet. All protein solutions were thoroughly flushed with nitrogen prior to the addition of trypsin and a nitrogen atmosphere was maintained during hydrolysis. After 60 min an additional 2% (w/w) trypsin was added. Hydrolysis was followed by measuring alkali consumption and was judged complete when no more NaOH was consumed (120 min). Simple calculation indicated quantitative cleavage of all the lysine and arginine peptide bonds of the protein.

Ion-exchange chromatography of the tryptic hydrolysate was performed as described by Hayashi *et al.* (1966). Aminex AG-1-X2 (minus 400 mesh) was packed to a depth of 93 cm in a 1 × 100 cm column and equilibrated with 0.2 M ammonium acetate (pH 7.2). The hydrolysate, representing 1 μmol of protein, was dissolved in 2 ml of buffer and applied to the column. After washing the column with 500 ml of initial buffer, a gradient was established between the starting buffer (500 ml) and 0.2 M acetic acid (1000 ml). This was followed by the stepwise change to 300 ml of 1 M, 300 ml of 2 M, and 200 ml of 8.3 M acetic acid. Fractions of 5 ml were collected at a flow rate of 60 ml/hr. Fractions corresponding to the peak region of each component absorbing at 280 nm were pooled and freeze-dried.

Precoated cellulose thin-layer plates were used for chromatography of peptides in pyridine–isoamyl alcohol–0.1 M NH_4OH (36:18:30, v/v). To detect peptides, chromatograms were sprayed with a 1% solution of ninhydrin in 90% ethanol containing 0.1% pyridine. To specifically identify tyrosine-containing peptides, chromatograms were further sprayed with a 0.1% solution of 1-nitroso-2-naphthol in 95% ethanol, followed by 20% nitric acid and then gentle heating for 3–5 min. Peptides containing tyrosine gave a pink-red color.

TABLE I: Extent of Tyrosine Modification in Ovotransferrin and Fe–Ovotransferrin with Tetranitromethane.

Protein	mol of Amino Acid/mol of Protein[a]			
	3-Nitrotyrosine		Tyrosine	mol of
	Spectral Anal.[b]	AA Anal.[c]	AA Anal.[c]	Fe/mol of Protein[d]
NO_2–ovotransferrin	20.1	13.3	0.5	0
NO_2Fe–ovotransferrin	13.8	5.9	6.9	1.8
RCM-NO_2Fe–ovotransferrin	13.5	5.7	7.0	0
Ovotransferrin	0	0	20	2.0

[a] Both spectral and amino acid analyses were performed after the removal of iron from modified protein derivatives. [b] The extent of 3-nitrotyrosine production was determined from the increase in absorbance at 428 nm for modified proteins in 0.1 M glycine–NaOH buffer (pH 10.5), using an extinction coefficient of 4.1×10^3 M^{-1} cm^{-1} for 3-nitrotyrosine and a mol wt of 80,000 for ovotransferrin derivatives. [c] Amino acid compositions were calculated using a mol wt of 80,000 for each protein. [d] The iron-binding capacity of each protein was determined by a procedure using "ferrozine" (Tsao *et al.*, 1974b).

Standard 3-nitrotyrosine did not produce a pink color under these conditions.

To determine the iron-binding capacity of modified Fe–ovotransferrin, a previously reported procedure employing "ferrozine" was used (Tsao *et al.*, 1974b).

The reaction of nitrated Fe–ovotransferrin with CNBr was conducted according to Phillips and Azari (1971). The only modification was increasing the reaction time from 5 to 24 hr. Also the separation of CNBr fragments on Bio-Gel P-150, acid–urea gel electrophoresis, and amino acid analysis were performed as described previously (Phillips and Azari, 1971).

Results

Nitration of Fe–ovotransferrin was attempted with the aim of producing a maximally nitrated derivative which would still contain unmodified tyrosines at the metal-binding sites. The feasibility of such an approach was indicated by producing a homogeneously nitrated preparation containing six–seven unmodified tyrosine residues, which retained full chromogenic activity. The results of the nitration of ovotransferrin and Fe–ovotransferrin are shown in Table I. By spectral analysis, nitration of iron-free ovotransferrin showed the quantitative conversion of all 20 tyrosines to 3-nitrotyrosines, concomitant with a complete loss of iron-binding activity. Under the same conditions, however, Fe–ovotransferrin showed the modification of only 14 tyrosines and almost complete retention of iron-binding activity. On the other hand, the extent of modification of tyrosine to 3-nitrotyrosine, as found from amino acid analysis of the modified ovotransferrin and Fe–ovotransferrin preparations, was 13 and 6 mol of nitrotyrosine/mol of protein, respectively. These values are 35 and 56% lower, respectively, than those determined by spectral analysis. Addi-

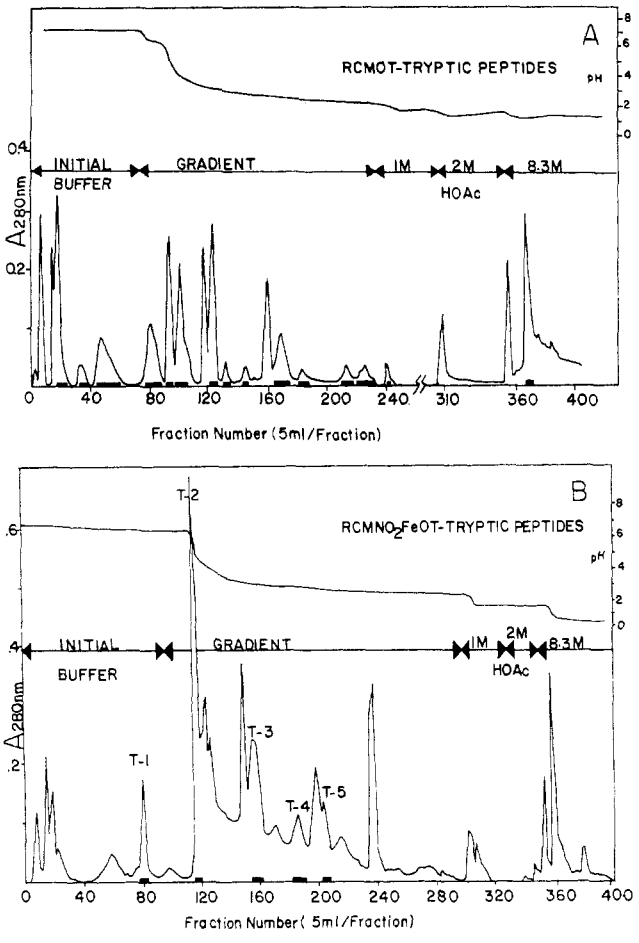

FIGURE 1: Chromatographic pattern of the tryptic peptides from RCM-ovotransferrin (A) and RCM-NO_2Fe-ovotransferrin (B). Approximately 1 μmol of reduced and carboxymethylated proteins was subjected to tryptic hydrolysis at 37° and subsequently applied to a 1 × 100 cm column of Aminex AG-1-X2 equilibrated in 0.2 M ammonium acetate (pH 7.2) (initial buffer). After washing the column with 500 ml of initial buffer, a gradient was established between the starting buffer (500 ml) and 0.2 M acetic acid (1000 ml). This was then followed by the stepwise change to 300 ml of 1 M, 300 ml of 2 M, and 200 ml of 8.3 M acetic acid as indicated by arrows on the diagram. Fractions of 5 ml were collected at a flow rate of 60 ml/hr and monitored for absorbance at 280 nm.

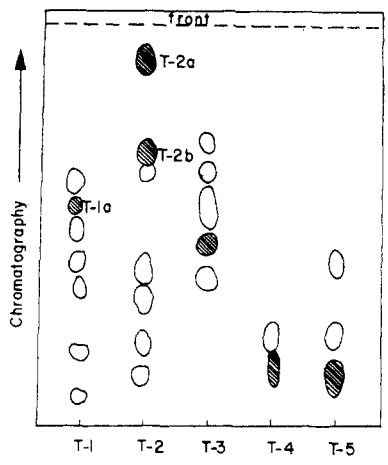

FIGURE 2: Thin-layer chromatography pattern of peptides obtained from the tryptic hydrolysis of RCM-NO_2Fe-ovotransferrin (Figure 1B). Chromatography was performed on a precoated microcrystalline cellulose plate in pyridine–isoamyl alcohol–0.1 M NH_4OH (36:18:30, v/v). Peptides were detected with ninhydrin (open spots); tyrosine-containing peptides were detected by reaction with 1-nitroso-2-naphthol (shaded spots).

tionally, NO_2–ovotransferrin shows a negligible amount of free tyrosine, whereas NO_2Fe–ovotransferrin shows 7 mol of unreacted tyrosines/mol of portein. It is also seen that NO_2Fe–ovotransferrin gave the same values for nitrotyrosine and tyrosine both before and after reduction and carboxymethylation.

Charge and molecular weight properties of the nitrated preparations were tested by gel electrophoresis in alkaline and acid–urea media, and by sedimentation analysis in the ultracentrifuge. Electrophoretic patterns for NO_2-ovotransferrin and NO_2Fe–ovotransferrin each showed predominantly one major protein band, which accounted for approximately 90% of the total protein. Sedimentation analysis showed only one sedimenting peak for each protein, resembling that of native ovotransferrin. These results indicated that nitration was suitable for producing nonaggregating nitrated protein molecules with very similar extents of modification. The NO_2Fe–ovotransferrin preparation, in addition, retained full chromogenic activity and possessed specific tyrosines, presumably those directly involved in the iron-binding sites, still unmodified. This, therefore, provided a further opportunity for identifying the particular segments of the ovotransferrin molecule containing these unmodified tyrosine residues.

Two approaches were employed for identifying the polypeptide segments containing unmodified tyrosines. In one approach, NO_2Fe–ovotransferrin was reduced and carboxymethylated and then subjected to hydrolysis with trypsin Figures 1A and 1B show the chromatographic separation of the tryptic peptides from RCM-ovotransferrin and RCM-NO_2Fe–ovotransferrin eluted from an Aminex AG-1-X2 column. The solid lines under the peaks indicate the fractions which gave a positive test for tyrosine by reaction with 1-nitroso-2-naphthol. At least 15 tyrosine-positive fractions were detected for the tryptic peptides from RCM-ovotransferrin. In contrast, the RCM-NO_2Fe–ovotransferrin tryptic digest showed only five major tyrosine-positive fractions, identified as T-1 to T-5. The thin-layer chromatographic (tlc) patterns for these five fractions are shown in Figure 2, where the shaded areas indicate tyrosine-positive peptides. The tyrosine peptides in fractions T-1, T-2, and T-3 showed a typical pink-red color with 1-nitroso-2-naphthol, whereas those of T-4 and T-5 showed an orange color, which apparently was due to the presence of 3-nitrotyrosine residues in these peptides (3-nitrotyrosine did not produce a pink-red color with 1-nitroso-2-naphthol reagent). The presence of six–seven unmodified tyrosines in NO_2Fe–ovotransferrin and the appearance of six tyrosine-positive peptides indicate that the tyrosines are apparently distributed equally among the six peptides. All the fractions, however, show various degrees of contamination with tyrosine-free peptides. In general, further purification of these peptides by different chromatographic procedures did not significantly improve the resolution of the tyrosine-containing peptides. In addition, the yields of tyrosine peptides were significantly reduced by these procedures. In one instance, however, it was possible to further purify the high R_F tyrosine-peptide (identified as T-1a in Figure 2) by repeated tlc procedures. This peptide was composed of 1 mol each of arginine, aspartic acid, threonine, serine, glutamic acid, leucine, tyrosine, and phenylalanine, 2 mol of alanine, and 5 mol of glycine.

In a second approach CNBr cleavage of NO_2Fe–ovotransferrin was performed in order to produce relatively larger

polypeptide fragments. Figure 3 presents the elution profile of the fragments from CNBr-cleaved NO_2Fe–ovotransferrin and also shows the rechromatography of both NO_2CF1 and NO_2CF2. In this way, NO_2CF1 and NO_2CF2 were each isolated in homogeneous form (as judged by acid–urea gel electrophoresis) and correspond to the nitrated derivatives of CF1 and CF2 isolated previously from CNBr-cleaved ovotransferrin (Tsao *et al.*, 1974a). The initial fraction, NO_2CF0, represented 35% of the total protein applied to the column. It showed one band in acid–urea gel electrophoresis and had approximately the same mobility as uncleaved NO_2Fe–ovotransferrin. The fragments denoted NO_2CF3 and NO_2CF4 were purified to homogeneity in preliminary studies as described previously (Tsao *et al.*, 1974a). However, since neither of these fragments contained free tyrosines (see below) they were not routinely rechromatographed as was done with NO_2CF1 and NO_2CF2.

Table II shows the amino acid composition of NO_2Fe–ovotransferrin before and after CNBr cleavage and of the individual fractions obtained from Bio-Gel P-150 chromatography. It is seen that the amino acid compositions of NO_2Fe–ovotransferrin and CNBr-cleaved NO_2Fe–ovotransferrin (mixture of fragments) are essentially the same. The exception is the presence of two unmodified methionines and a total of six residues of homoserine–homoserine lactone for CNBr-cleaved NO_2Fe–ovotransferrin, as compared to eight methionines for the uncleaved protein. It must be pointed out that the amino acid composition of NO_2Fe–ovotransferrin (with the exception of tyrosine and tryptophan) was also the same as for unmodified ovotransferrin. The tryptophan content of the

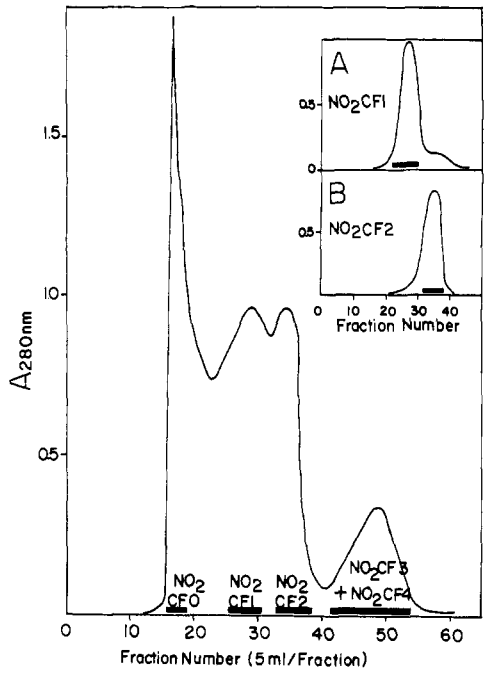

FIGURE 3: Fractionation of CNBr-treated NO_2Fe–ovotransferrin on Bio-Gel P-150. A 1.8 × 120 cm column of Bio-Gel P-150 was used, with 1 M propionic acid as the eluent. Fractions of 5 ml were collected at a flow rate of 15 ml/hr. Horizontal bars indicate fractions pooled. The insert presents the rechromatography of NO_2CF1 (A) and NO_2CF2 (B), empolying the same column of Bio-Gel P-150.

TABLE II: Amino Acid Composition of Nitrated Iron–Ovotransferrin and Its CNBr Fragments.

	No. of Residues/Molecule[a]							
AA	NO_2Fe–Ovotransferrin	CNBr + NO_2Fe–Ovotransferrin	NO_2CF0[b]	NO_2CF1	NO_2CF2	NO_2CF3	NO_2CF4	Total
Asp	73.9	73.8	67.1	34.5	22.8	6.3	12.6	76.2
Thr	34.2	33.6	33.9	18.4	12.8	1.9	6.2	39.3
Ser	40.8	41.3	40.0	20.7	14.0	1.7	6.4	42.8
Glu	67.8	65.9	62.0	32.2	20.7	5.9	9.0	67.8
Pro	27.8	27.2	26.0	13.8	8.8	1.9	3.5	28.0
Gly	51.0	51.4	52.2	29.9	18.3	2.3	10.6	61.1
Ala	56.1	56.3	53.9	23.7	17.2	2.9	7.2	51.0
Half-Cys	32.0	33.7	23.1	13.5	11.9	1.6	7.0	34.0
Val	49.4	49.8	44.4	23.0	15.0	4.4	11.9	54.3
Met	8.1	2.0	0	Trace	Trace	0	Trace	0.6
Ile	26.4	26.8	25.8	12.4	6.9	1.0	5.3	25.6
Leu	50.2	49.8	44.3	26.0	15.7	2.5	5.8	50.0
Tyr	6.9	6.8	4.0	4.4	3.5	0	0.2	8.1
Phe	28.3	27.1	24.0	13.8	9.9	1.7	1.6	27.0
3-NO_2Tyr	5.7	5.6	5.2	3.0	1.8	0	1.2	6.0
His	9.9	10.7	9.9	5.4	3.7	0.8	0.01	9.9
Lys	54.8	53.1	47.4	23.0	17.5	4.0	10.4	54.9
Arg	33.9	33.2	28.1	14.6	9.4	4.9	4.9	33.8
Homoserine–homoserine lactone	0	6	4.2	2.1	1.9	1.0	2.1	7.1

[a] Compositions were calculated on the basis of the following molecular weights: NO_2Fe–ovotransferrin, NO_2Fe–ovotransferrin + CNBr (mixture of all fragments), and NO_2CF0, 76,000; NO_2CF1, 36,000; NO_2CF2, 23,000; NO_2CF3, 5000; NO_2CF4, 12,000; based on previous data (Tsao *et al.*, 1974a). [b] This is the first protein peak which is eluted from the Bio-Gel chromatography of the CNBr–NO_2Fe–ovotransferrin reaction mixture. It constituted approximately 35% of the initial protein.

TABLE III: Tyrosine and 3-Nitrotyrosine Content of CNBr Fragments from NO_2Fe–Ovotransferrin.

Protein	mol of Amino Acid/mol of Protein		
	3-Nitrotyrosine		
	Spectral[a] Anal.	AA Anal.	Tyrosine AA Anal.
NO_2CF1	6.8	3.0	4.4 (12)[b]
NO_2CF2	2.5	1.9	3.6 (6)
NO_2CF3	0.25	0	0 (0)
NO_2CF4	1.7	1.1	0.20 (2)
Total	11.3	6.0	8.2 (20)

[a] The procedure for the calculation of 3-nitrotyrosine content was that of Table I, using the molecular weights for each fragment as given in Table II. [b] The values in parentheses represent the tyrosine content of the CNBr fragments (CF1, CF2, CF3, and CF4) obtained from unmodified ovotransferrin (Tsao *et al.*, 1974a).

NO_2Fe–ovotransferrin was found to be 3 residues per molecule, as compared to 12 residues for unmodified ovotransferrin.

The composition of NO_2CF0 shows a similarity to CNBr-cleaved NO_2Fe–ovotransferrin (mixture). However, this fraction contains no methionine and smaller amounts of half-cystine, tyrosine, 3-nitrotyrosine, and homoserine–homoserine lactone as compared to CNBr-cleaved NO_2Fe–ovotransferrin. Of greatest interest, however, was the finding of free tyrosine in only NO_2CF1 and NO_2CF2. These two fragments, therefore, must have originated from protected regions of the native Fe–ovotransferrin molecule. The sum of the amino acid composition of CNBr fragments corresponds to the composition of uncleaved NO_2Fe–ovotransferrin within the experimental error of $\pm 5\%$.

Table III presents a summary of the nitrotyrosine and tyrosine content of the CNBr fragments, as determined by different procedures. The values in parentheses represent the tyrosine content of the CNBr fragments obtained from unmodified ovotransferrin, as reported previously (Tsao *et al.*, 1973a). Again, no free tyrosines are found for either NO_2CF3 or NO_2CF4. In contrast, NO_2CF1 and NO_2CF2 show 5 and 4 mol of unmodified tyrosine/mol, respectively. In general, lower recoveries of 3-nitrotyrosines were found by amino acid analysis of the fragments, as compared to the values found by spectrophotometry. The sum of nitrotyrosines plus the unmodified tyrosines for each fragment, however, accounted closely for the tyrosine content of each of the unmodified fragments.

Discussion

The present investigation was based on the known difference in reactivity of ovotransferrin and Fe–ovotransferrin to chemical modifications, particularly in respect to tyrosine residues (Komatsu and Feeney, 1967; Line *et al.*, 1967; Phillips and Azari, 1972; Teuwissen *et al.*, 1973). Hence, nitration of Fe–ovotransferrin with tetranitromethane was used in this study to modify those tyrosines not directly involved in iron-binding activity. Thirteen–fourteen tyrosine residues (out of 20) could be modified with no loss of iron-binding activity. Therefore, tyrosines not nitrated (6–7 out of 20 residues) are apparently involved in some capacity in the chelation of iron, some presumably as ligands for the metal. This contention is supported by results obtained by further nitrating NO_2Fe–ovotransferrin after removal of iron. Such a second nitration was accompanied by a complete loss of iron-binding activity. In view of the involvement of four tyrosine residues in the two iron-binding sites of ovotransferrin (Wishnia *et al.*, 1961; Tan and Woodworth, 1969; Luk, 1971; Phillips and Azari, 1972), the finding of six–seven unmodified tyrosines in NO_2Fe–ovotransferrins indicates that two–three of the unmodified residues are not directly involved in metal binding.

The reaction of proteins with tetranitromethane results primarily in the nitration of tyrosine residues, although other reactions have been shown to occur (Riordan and Vallee, 1972). For instance, the formation of intermolecular cross-links, presumably through tyrosines, has been reported for insulin (Boesel and Carpenter, 1970), collagen and immunoglobulin G (Doyle *et al.*, 1968), and the model peptide, glycyl-L-tyrosine (Boyd and Smith, 1971). In the present study, however, intermolecular cross-linking of NO_2Fe–ovotransferrin molecules was ruled out, since high molecular weight components were not observed in ultracentrifugation experiments. Similar results were reported for nitrated human lactoferrin (Teuwissen *et al.*, 1973). On the other hand, intramolecular cross-linking remains a possibility, especially in light of compositional data for the CNBr product noted as NO_2CF0. This product, with no methionines, possessed 4 mol of homoserine–homoserine lactone/mol of protein. Previous work on the CNBr cleavage of ovotransferrin has demonstrated that conversion of methionine to homoserine–hemoserine lactone results, in all cases, in cleavage of the peptide bond containing the methionine residue (Phillips and Azari, 1971; Tsao *et al.*, 1974a). Therefore, the presence of four homoserine–homoserine lactone residues in NO_2CF0 should have been indicative of the presence of four–five polypeptide chains in the CNBr-cleaved NO_2CF0 and should have produced several protein bands in acrylamide gels. NO_2CF0, however, gave only a single band in acrylamide gel electrophoresis before and after reduction and carboxymethylation. These data, therefore, indicate that intramolecular cross-linking between CNBr fragments in NO_2CF0 is a strong possibility.

Tetranitromethane has also been shown to react with methionine and tryptophan (Riordan and Vallee, 1972), both substitution and oxidation reactions being reported. Our concern has been particularly with the oxidative modification of methionine, since peptide bonds contributed by oxidation products of methionine, such as methionine sulfoxide, are known to be resistant to CNBr cleavage (Gross, 1967). Of interest in this regard is the apparent finding of two methionine residues in the acid hydrolysate of CNBr-cleaved NO_2Fe–ovotransferrin (*i.e.*, the mixture of all fragments). Acid hydrolysis would generate free methionine from any methionine sulfoxide present (Means and Feeney, 1971). Another source of concern was the lower recovery for the sum of tyrosine and mononitrotyrosine residues which reached about 65% of the total tyrosine content (20 residues) of native ovotransferrin and Fe-ovotransferrin. In a separate study, we found that acid hydrolysis of standard tyrosine and mononitrotyrosine, separately or mixed, did not result in the destruction of these compounds. Therefore, the lower recovery found in the present study is apparently not due to the result of acid hydrolysis of modified protein. Although the precise reasons for low recoveries are unknown several alternatives do exist which may relate to the inherent reaction of tetranitromethane with proteins. These may involve intra-

molecular cross-linking through tyrosines, formation of nitro derivatives other than mononitrotyrosine, and oxidative modifications.

The lower recovery of 3-nitrotyrosine by amino acid analysis as compared to spectrophotometric determination (from the increase in absorbance at 428 nm) found in the present investigation has also been reported previously by Boyd and Smith (1971) and by Williams and Lowe (1971). It has generally been assumed that the increase in absorbance at 428 nm is due to the yellow 3-nitrotyrosine residues. However, other possible reaction products, such as 3,5-dinitrotyrosine, nitrotryptophan, and various tryptophan oxidation products, may also contribute to the visible absorbance spectrum of the nitrated proteins (Teuwissen *et al.*, 1973). The presence of 3,5-dinitrotyrosine, in the hydrolysis product of nitrated ovotransferrin, was indicated by the appearance of a small elution peak (preceding methionine) which corresponded to the position of authentic 3,5-dinitrotyrosine. The destruction of tryptophan was also indicated by the recovery of only 25% of expected tryptophan from the nitrated protein. Similar results were reported by Teuwissen *et al.* (1973) for nitrated human lactoferrin. In addition, these authors demonstrated the formation of several colored products from nitration of free tryptophan, one of which was attributed to nitrotryptophan. In the present investigation we have also observed the appearance of several small elution peaks in the amino acid elution profile of nitrated proteins. The identity of these peaks remains to be investigated.

Attempts to separate and purify individual tyrosine-containing tryptic peptides from NO_2Fe–ovotransferrin by conventional chromatographic procedures were not successful. This was primarily due to the inherent difficulty of separating a very few tyrosine peptides from a much larger number of unwanted peptides. Nonetheless, it was encouraging to find that the number of tyrosine-positive peptide fractions was reduced from 15 for RCM-ovotransferrin to 5 for RCM-NO_2-Fe–ovotransferrin. This indicated more directly the feasibility of nitrating Fe–ovotransferrin to produce a homogeneously modified protein still possessing unmodified tyrosines from the metal-binding sites. Also, it appears that the six unmodified tyrosines are equally distributed among the six tyrosine-positive tryptic peptides observed by tlc.

Of more interest was the observation that CNBr cleavage of NO_2Fe–ovotransferrin was successful in cleaving approximately 65% of the protein to produce nitrated polypeptide fragments which possessed the molecular weight and composition of the CNBr fragments produced from unmodified ovotransferrin (Tsao *et al.*, 1974a). The absence of free tyrosine in NO_2CF3 and NO_2CF4 clearly indicated that these fragments do not contribute tyrosines involved in metal binding. On the other hand, the presence of free tyrosines in NO_2CF1 and NO_2CF2 does indicate their participation in iron-binding activity. Indeed, the five unmodified tyrosines of NO_2CF1 and the four tyrosines of NO_2CF2 more than account for the four tyrosine residues believed to be necessary for the binding of two iron atoms by native ovotransferrin. Hence, each of these fragments may contain essential and/or otherwise protected tyrosines. Although this situation requires clarification, one thing appears certain. Since CF1 has previously been shown to bind specifically 1 mol of iron/mol of fragment (Tsao *et al.*, 1974b), at least two essential iron-binding tyrosines must reside within the iron-binding site of this fragment. The assignment of two additional tyrosines for the second iron-binding site, however, is not possible at present. These two residues may reside completely on CF2 or on both CF1 and CF2; further studies are necessary to clarify this situation. Finally, it is of interest that CNBr cleavage of nitrated human serum iron transferrin (Bezkorovainy and Grohlich, 1972) produced two fractions which contained unmodified tyrosine residues, presumably from the iron-binding sites.

References

Aasa, R. (1972), *Biochem. Biophys. Res. Commun. 49*, 806.

Aasa, R., Malmstrom, B. G., Saltman, P., and Vanngard, T. (1963), *Biochim. Biophys. Acta 75*, 203.

Azari, P., and Baugh, R. F. (1967), *Arch. Biochem. Biophys. 138*, 32.

Azari, P., and Feeney, R. E. (1961), *Arch. Biochem. Biophys. 92*, 44.

Bezkorovainy, A., and Grohlich, D. (1972), *Biochim. Biophys. Acta 263*, 645.

Boesel, R. W., and Carpenter, F. H. (1970), *Biochem. Biophys. Res. Commun. 38*, 678.

Boyd, N. D., and Smith, D. B. (1971), *Can. J. Biochem. 49*, 154.

Doyle, R. J., Belle, J., and Roholt, O. A. (1968), *Biochim. Biophys. Acta 160*, 274.

Feeney, R. E., and Komatsu, S. K. (1966), *Struct. Bonding (Berlin) 1*, 149.

Gross, E. (1967), *Methods Enzymol. 11*, 278.

Hayashi, K., Shimoda, T., Imoto, T., and Fumatsu, M. (1966), *J. Biochem. (Tokyo) 64*, 365.

Komatsu, S. K., and Feeney, R. E. (1967), *Biochemistry 6*, 1136.

Line, W. F., Grohlich, D., and Bezkorovainy, A. (1967), *Biochemistry 6*, 3393.

Luk, C. K. (1971), *Biochemistry 10*, 2838.

Means, G. E., and Feeney, R. E. (1971), Chemical Modification of Proteins, San Francisco, Calif., Holden-Day, p 164.

Phillips, J. L., and Azari, P. (1971), *Biochemistry 10*, 1160.

Phillips, J. L., and Azari, P. (1972), *Arch. Biochem. Biophys. 151*, 445.

Riordan, J. F., and Vallee, B. L. (1972), *Methods Enzymol. 25*, 515.

Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1966), *Biochemistry 5*, 3582.

Tan, A. T., and Woodworth, R. C. (1969), *Biochemistry 8*, 3711.

Teuwissen, B., Masson, P. L., Osinski, P., and Hermans, J. F. (1973), *Eur. J. Biochem. 35*, 366.

Tsao, D., Azari, P., and Phillips, J. L. (1974a), *Biochemistry 13*, 397.

Tsao, D., Morris, D. H., Azari, P., Tengerdy, R. P., and Phillips, J. L. (1974b), *Biochemistry 13*, 403.

Williams, J., and Lowe, J. M. (1971), *Biochem. J. 121*, 203.

Wishnia, A., Weber, I., and Warner, R. C. (1961), *J. Amer. Chem. Soc. 83*, 2071.

Woodworth, R. C., Morallee, K. G., and Williams, R. J. P. (1970), *Biochemistry 9*, 839.