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On the Structure of Ovotransferrin. I. Isolation and Characterization of Cyanogen Bromide Fragments. Reevaluation of the Primary Structure†

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ABSTRACT: Cyanogen bromide cleavage of ovotransferrin, an iron-binding protein from chicken egg white, followed by reduction and carboxymethylation of each initially liberated fragment, produced a total of eight distinct polypeptides, ranging in molecular weight from 2700 to 35,000 daltons. The total molecular weight of the polypeptides accounts for the molecular weight of the native protein. Additionally, 1 mol of free homoserine–homoserine lactone was produced, indicating the existence of a single methionylmethionine sequence in the uncleaved protein. All eight methionines of ovotransferrin could be accounted for by the cyanogen bromide reaction products. These data indicate that, on the basis of CNBr fragmentation, the primary structure of ovotransferrin is not duplicated.

The transferrins are a group of homologous iron-binding glycoproteins which are widely distributed in various biological fluids (Feeney and Komatsu, 1966). Because of their high molecular weight (*ca.* 80,000), it has been suggested that transferrins may possess a subunit structure (a dimer). Indeed, Jeppsson (1967) has reported that human transferrin exists as a dimer with the monomer possessing a mol wt of 40,000. However, this work has been repudiated by several groups (Bezkorovainy and Grohlich, 1967; Greene and Feeney, 1968; Mann *et al.*, 1970), who have demonstrated conclusively that transferrins are not dimeric but consist of a single polypeptide chain. The possibility of a duplicated internal structure has also been suggested from a variety of data. Electron spin resonance measurements indicated the presence of two equivalent metal-binding sites (Windle *et al.*, 1963; Aisen *et al.*, 1966). The isolation of two identical carbohydrate chains from human serum transferrin by Jamieson (1965) also pointed to the possibility of a molecule composed of duplicate segments. Furthermore, tryptic digests of both rabbit transferrin (Baker *et al.*, 1968) and human transferrin (Jeppsson, 1967) appeared to give fewer spots on peptide maps than would be expected from their arginine and lysine contents. A more direct form of evidence, however, reported by this laboratory, showed that ovotransferrin (also known as conalbumin) released a maximum of four fragments by CNBr cleavage, while twice that number should have been expected (Phillips and Azari, 1971). In addition, the total molecular weight of the four fragments was found to be one-half that of the native protein. On the basis of these results, Phillips and Azari (1971) suggested a duplicated primary structure for ovotransferrin.

On the other hand, there is some evidence that conflicts with the duplicate structure model for the transferrins. For instance, Elleman and Williams (1970) have identified 34 unique cysteic acid containing peptides from performic acid oxidized ovotransferrin (ovotransferrin contains 32 half-cystine residues per molecule). Also, only one carbohydrate chain per molecule of both bovine serum transferrin (Hudson *et al.*, 1973) and chicken serum transferrin (Williams, 1968) has been reported. More recently, Bezkorovainy and Grohlich (1972) have isolated a total of six fragments from CNBr-treated human serum transferrin. The total molecular weight of these fragments accounted for the entire molecular weight of the native protein. Additionally, both reinterpretation of electron paramagnetic resonance (epr) spectra (Aasa, 1972) and the spectroscopic characteristics of a series of mixed metal–ovotransferrin complexes (Aisen *et al.*, 1973) provide evidence that the two metal-binding sites of ovotransferrin are nonidentical.

Because of these conflicting reports, we have undertaken a reinvestigation of the cyanogen bromide cleavage of ovotransferrin. Emphasis has been placed on the characterization of cyanogen bromide fragments, particularly with respect to molecular weight, composition, and the presence of

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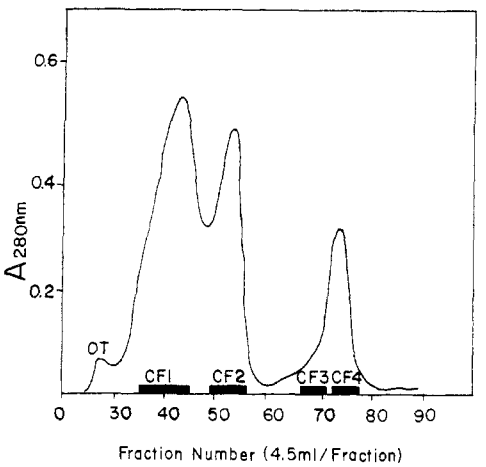

FIGURE 1: Fractionation of CNBr-treated ovotransferrin on Bio-Gel P-150. A 2.5 × 100 cm column of Bio-Gel P-150 was used, with 1 M propionic acid as eluent. Fractions of 4.5 ml were collected at a flow rate of 20 ml/hr. Horizontal bars indicate fractions pooled.

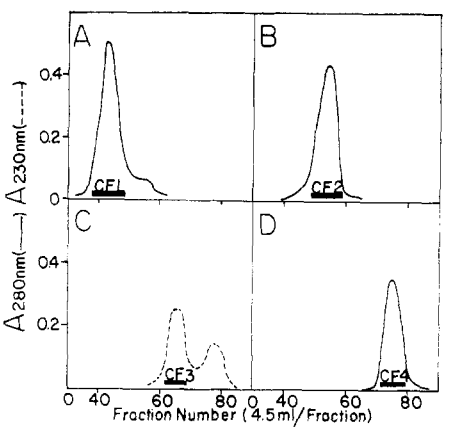

FIGURE 2: Rechromatography of pooled fractions from Figure 1. Fractionation conditions were the same as for Figure 1, with the exception of CF3 which was eluted with 0.1 M NH_4HCO_3 (pH 8.5).

more than one polypeptide[1] constituting each fragment. Evidence is now presented which indicates that the primary structure of ovotransferrin is not composed of duplicated CNBr fragments, as was suggested previously (Phillips and Azari, 1971).

Experimental Procedure

Materials. Ovotransferrin used was a 4× crystallized preparation, isolated as described previously (Azari and Baugh, 1967). The protein appeared homogeneous by sedimentation velocity and by acrylamide gel electrophoresis in both acid and alkaline buffer systems.

Bio-Gel P-150 (100–200 mesh) and Bio-Gel P-60 (200–400 mesh) were obtained from Bio-Rad Laboratories. Sephadex G-50 (40–120 μ) was a product of Pharmacia. Cyanogen bromide was an Eastman Kodak reagent. Propionic acid (buffer grade) was the product of Pierce Chemical Co. Dansyl amino acids and protein standards were purchased from Sigma Chemical Co. Precoated cellulose thin-layer plates (Avicel microcrystalline cellulose) were a product of Analtech, Inc. Polyamide plates were obtained from Cheng Chin Trading Company Ltd., Taiwan. Spectrapor 3 membrane tubing (mol wt cut-off 3500 daltons) was purchased from Spectrum Industries. All other reagents were of the highest grade available, and were used without further purification.

CNBr Cleavage. The reaction of ovotransferrin with CNBr was performed essentially according to procedures of Phillips and Azari (1971) and Tsao *et al.* (1974). The latter procedure was especially useful for obtaining a relatively more soluble preparation of CNBr fragments.

Chromatography of CNBr Fragments with Bio-Gel P-150. Bio-Gel P-150 was allowed to swell at least 48 hr at room temperature in 1 M propionic acid, and then packed to a depth of 90 cm in a 2.5 × 100 cm column. The column was equilibrated with 1 M propionic acid for 48 hr at a constant flow rate of 20 ml/hr. Samples of CNBr-treated ovotransferrin (50–60 mg) were dissolved in 3 ml of 1 M propionic acid and applied to the column. Fractions of 4.5 ml were collected, and the absorbance of each was measured at 280 nm in a Coleman 111 spectrophotometer. Appropriate fractions were pooled and freeze-dried. Further purification of pooled fractions was accomplished by rechromatography on the same column.

Acrylamide Gel Electrophoresis. The conditions for vertical slab acrylamide gel electrophoresis have been described previously (Phillips and Azari, 1971).

Amino Acid Analysis. Samples were hydrolyzed in glass-distilled 6 N HCl at 110° for 24 hr in sealed, evacuated tubes. The acid was removed by evaporation under vacuum. Amino acid analyses were performed essentially as described by Spackman *et al.* (1958) on a Beckman 120B amino acid analyzer employing a single column system and operated under accelerated conditions. A temperature gradient from 47 to 58° was necessary for good resolution of homoserine and homoserine lactone peaks from closely eluting amino acids.

N-Terminal Determination. N-Terminal amino acids were determined by the dansylation procedure of Gray (1972). Dansyl amino acids were identified by thin-layer chromatography on polyamide plates in the solvent system benzene–acetic acid (9:1, v/v). Standard dansyl amino acids were run for comparison.

Also, proteins were allowed to react with phenyl isothiocyanate according to Edman and Sjoquist (1956). The resulting phenylthiohydantoin amino acids were hydrolyzed in glass-distilled 6 N HCl at 150° for 24 hr in sealed evacuated tubes. The free amino acids thus generated were identified by amino acid analysis as described above.

Reduction and carboxymethylation of the CNBr fragments was conducted by the procedure recommended by Konigsberg (1972). A solution of each fragment was made to a final concentration of 1% in pH 8.6 buffer (0.5 M Tris–6 M guanidine·HCl–0.002 M EDTA). After an incubation time of 30 min at 50° under nitrogen, dithioerythritol was added to provide a 20-fold molar excess over disulfide content of ovotransferrin. The mixture was incubated in nitrogen atmosphere for 5 hr. Recrystallized iodoacetic acid, dissolved in 0.1 N NaOH, was then added to provide a 50-fold molar excess over disulfide content, and the solution was kept at room temperature in the dark for 30 min. After this time, the nitroprusside test for thiol groups was negative. The reaction mixture was finally chromatographed on either a Sephadex G-50 or a Bio-Gel P-60 column equilibrated with 0.1 M NH_4HCO_3 (pH 8.5).

[1] The "CNBr fragments" are designated as those fragments which are initially released from ovotransferrin by the action of CNBr. Further cleavage of CNBr fragments at the disulfide bonds results in the liberation of additional smaller fragments which are designated as "polypeptides."

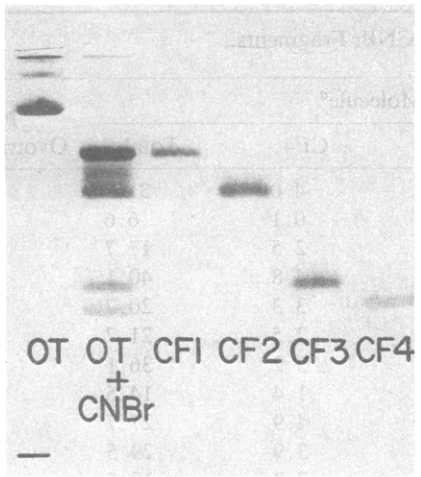

FIGURE 3: Acrylamide gel electrophoresis pattern of the purified CNBr fragments from ovotransferrin. The gel composition was 7% cyanogum-41, 0.12 M Tris-citrate buffer (pH 2.9), and 3 M urea. The electrode buffer was 0.3 M glycine–citrate (pH 4). Runs were made at 350 V (120 mA) for 2 hr at 1°. Protein bands were stained with Amido Black and destained electrophoretically.

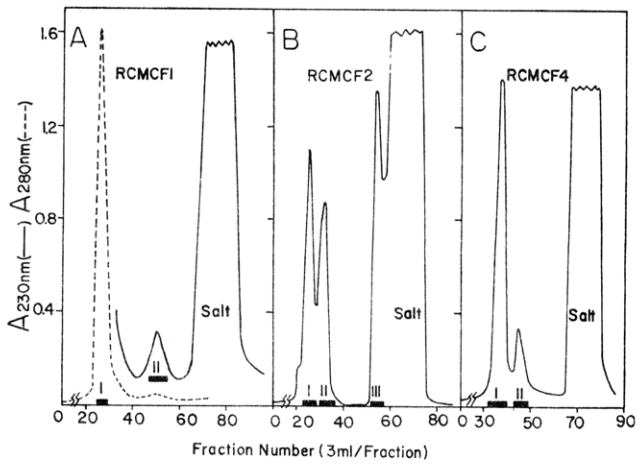

FIGURE 4: Chromatographic separation of the reduced and carboxymethylated components from the original CNBr fragments. The eluent was 0.1 M NH_4HCO_3 (pH 8.5) in all cases. Effluent was monitored for absorbance at 280 or 230 nm. Horizontal bars indicate fractions pooled. Figure 4A shows the elution pattern of polypeptide components of RCMCF1. Fractionation was performed on a 7.2 × 90 cm column of Sephadex G-50 (fine) at a flow rate of 30 ml/hr. Figure 4B shows the elution profile of polypeptide components from RCMCF2 from a 2.2 × 60 cm column of Bio-Gel P-60 at a flow rate of 30 ml/hr. Further purification of fractions I and II from RCMCF2 was achieved by rechromatography on the same column. Fraction III was rechromatographed on a 1 × 100 cm column of Sephadex G-25 (super fine) to remove salt. Figure 4C represents the fractionation profile of polypeptide components from RCMCF4. Gel chromatography was performed under the same conditions described above for RCMCF1.

Immunochemical Studies. Antigenic properties of the CNBr fragments were tested by double radial diffusion in agar against antibody specific for ovotransferrin, essentially as described by Azari and Phillips (1970).

Results

Amino acid analysis of CNBr-cleaved ovotransferrin showed that the conversion of methionine to homoserine and homoserine lactone was 95% complete. The elution pattern of the CNBr fragments from Bio-Gel P-150 is presented in Figure 1. The initial component was identified by amino acid analysis as uncleaved ovotransferrin and represented 2% (by weight) of the material applied. The dark horizontal bars under each subsequent peak indicate fractions pooled which predominantly contained fragments identified as CF1, CF2, CF3, and CF4. The decision for pooling various fractions was based on acrylamide gel electrophoresis of fractions along the elution profile. The total material contained within all the pooled fractions was approximately 94% (by weight) of the protein applied to the column.

Figures 2A, B, and D show the elution profiles for the rechromatography of fractions (from Figure 1) containing CF1, CF2, and CF4, respectively. For these fragments, rechromatography was accomplished on the same column of Bio-Gel P-150 with 1 M propionic acid as the eluent. With the pooled fraction containing CF3, however, different conditions for rechromatography had to be employed. Preliminary data on the amino acid composition of CF3 revealed the absence of tyrosine, thus making CF3 difficult to detect in the column effluent by measuring absorbance at 280 nm. Hence, CF3-containing fractions were rechromatographed on a column of Bio-Gel P-150 as before, but with a bicarbonate buffer (low absorbance at 230 nm) as the eluent instead of propionic acid (high absorbance at 230 nm). CF3 was then detected in the column effluent by measuring the absorbance at 230 nm, resulting in the elution profile of Figure 2C.

In all cases, the second chromatography was sufficient to resolve each major fragment (for any given pooled fraction of Figure 1) from the small amount of contaminating fragment. Hence, the fractions of Figures 2A–D indicated by dark horizontal bars were found to each contain a homogeneous fragment, as judged by the appearance of one band in acid–urea acrylamide gel electrophoresis (Figure 3). Similar results were also obtained with sodium dodecyl sulfate–gel electrophoresis in the absence of mercaptoethanol.

Table I presents the amino acid composition of the four CNBr fragments, calculated on the basis of one residue of homoserine–homoserine lactone for each fragment. The total amino acid composition and the sum of minimum molecular weights of the four fragments account for approximately one-half the total number of amino acids and one-half the molecular weight of the native protein. The carbohydrate content of the fragments is also presented in Table I and shows that fragments CF1 and CF2 contain both mannose and glucosamine while CF3 and CF4 are devoid of carbohydrate. The total carbohydrate content of the fragments also is one-half that of native ovotransferrin. These results are similar to those reported earlier (Phillips and Azari, 1971).

In a preliminary experiment, the possibility of more than one polypeptide chain comprising each major fragment was tested by sodium dodecyl sulfate–gel electrophoresis, after reduction of the fragments by 2-mercaptoethanol. The results obtained indicated that both CF2 and CF4 were each cleaved into at least two components after reduction, while CF1 and CF3 were apparently unaffected. In order to investigate further the subunit structure of the CNBr fragments, each fragment was reduced and carboxymethylated and then chromatographed on columns of Sephadex G-50 or Bio-Gel P-60. In this way, the individual polypeptide chains comprising each fragment could be isolated and characterized. Figures 4A, B, and C present the elution profiles for polypeptides: RCMCF1, RCMCF2, and RCMCF4, respectively (RCM, reduced and carboxymethylated). Each fragment is seen to contain more than one component. The regions indicated by the dark hori-

TABLE I: Amino Acid and Carbohydrate Composition of Ovotransferrin and Its CNBr Fragments.

Amino Acid	No. of Residues/Molecule[a]					Native Ovotransferrin[b]
	CF1	CF2	CF3	CF4	Total	
Lys	14.3	9.4	4.2	4.0	31.9	56
His	3.8	2.0	0.7	0.1	6.6	12
Arg	5.7	4.8	4.7	2.5	17.7	28
Asp	17.3	11.8	6.2	4.8	40.1	72
Thr	9.0	6.9	1.5	3.3	20.7	33
Ser	9.7	8.0	1.5	2.5	21.7	40
Glu	13.2	11.0	6.7	5.2	36.1	67
Pro	7.8	4.2	1.1	1.4	14.5	26
Gly	11.9	9.1	1.8	4.9	27.7	52
Ala	15.0	7.8	2.8	3.9	29.5	55
Half-Cys	8.3	5.8	2.1	2.3	18.5	31
Val	10.5	7.0	4.2	4.9	26.6	52
Met	0	0	0	0	0	8
Ile	7.4	3.1	0.9	2.1	13.5	24
Leu	10.9	7.0	2.9	3.7	24.5	49
Tyr	4.8	3.2	0.3	0.9	9.2	20
Phe	5.9	4.8	1.6	1.0	13.3	26
Homoserine + homoserine lactone	1	1	1	1	4	0
Trp[c]	3.0	1.2	0	1.9	6.1	12
Minimum mol wt[d]	17,479	12,333	5173	5331	40,316	76,600
Mannose (mol/mol)	1	1	0	0	2	4
Glucosamine (mol/mol)	1	2	0	0	3	6

[a] Average of duplicate determinations, accuracy ±5%. Assuming one residue of homoserine + homoserine lactone per fragment. [b] From a previous investigation (Azari and Baugh, 1967). [c] Determined colorimetrically with *p*-dimethylaminobenzaldehyde (Azari and Phillips, 1970). The number of residues per molecule was based on the minimum mol wt for each fragment as shown in the table. [d] Estimated from the amino acid composition data assuming one residue of homoserine–homoserine lactone per each fragment.

zontal bars indicate fractions which were pooled, freeze-dried, and then rechromatographed on the same column of Sephadex or Bio-Gel. By this procedure, a sufficient amount of each polypeptide in homogeneous form was isolated for characterization. Homogeneity was assessed by sodium dodecyl sulfate–gel electrophoresis (for polypeptides of mol wt >10,000) and by high voltage electrophoresis (for smaller mol wt polypeptides, particularly RCMCF1-II and RCMCF2-III, which were undetectable by gel electrophoresis). Hence, RCMCF1 was separated into two polypeptides, RCMCF2 into three polypeptides, and RCMCF4 into two polypeptides. CF3 was unaffected by reduction and carboxymethylation, in contrast to the other CNBr fragments. CF3, therefore, is comprised of a single polypeptide with one intrachain disulfide bond.

Table II shows the amino acid composition of each homogeneous reduced and carboxymethylated polypeptide from the original CNBr fragments, calculated on the basis of one residue of homoserine–homoserine lactone per polypeptide. The only exception was RCMCF2-III, which does not contain homoserine. Its composition was determined on the basis of one residue of carboxymethylcysteine. The mol wt of 74,691 shown for the total of all polypeptides is in line (within the experimental error of ±5%) with the mol wt of 76,600 for native ovotransferrin (Azari and Baugh, 1967). There was, however, still the possibility that the reaction of ovotransferrin with CNBr produced a very small peptide(s) or free amino acid(s), which would be undetected by the procedures employed. We have, therefore, employed two separate approaches for investigating this possibility. In one, ice-cold 10% Cl_3CCOOH was added to an aqueous solution of the CNBr fragments to a final concentration of 5%. The precipitate was centrifuged and the supernatant was concentrated *in vacuo* over sodium hydroxide. In the second approach, the reaction mixture containing CNBr fragments was dialyzed in small-pore dialysis tubing (mol wt cut-off 3500 daltons) and the dialysate was concentrated by freeze-drying. Qualitative cellulose thin-layer chromatography (tlc) of both concentrated fractions in pyridine–isoamyl alcohol–0.1 M NH_4OH (36:18:30, v/v) showed two major ninhydrin positive spots, corresponding to homoserine and its lactone. Quantitative amino acid analysis of the concentrated fractions revealed the production of 1 mol of homoserine–homoserine lactone per mol of CNBr-treated ovotransferrin. No significant amounts of other amino acids were detected. It thus appears that one methionyl-methionine sequence is present in the primary structure of native ovotransferrin.

Table III shows the N-terminal amino acids for both the intact CNBr fragments and their constituent polypeptide chains. The identity of each N-terminal amino acid was based on both the identification of dansyl amino acids by tlc and by the determination of the free amino acids generated from phenylthiohydantoin amino acids. The presence of only a single N-terminal amino acid for each polypeptide is additional evidence for their apparent homogeneity.

The double radial immunodiffusion pattern of ovotransfer-

TABLE II: Amino Acid Composition of the Polypeptides Obtained from Reduction and Carboxymethylation of the CNBr Fragments.

	No. of Residues/Molecule								
	RCMCF1-		RCMCF2-				RCMCF4-		
Amino Acid	I	II	I	II	III	RCMCF3	I	II	Total
Lys	25.1	2.4	8.2	3.8	2.6	4.2	6.9	1.2	54.4
His	6.7	0.3	3.7	0.8	1.1	0.7	0	0.2	13.5
Arg	10.9	1.3	4.5	3.9	0.8	4.7	5.8	0.4	32.3
CM-Cys	11.1	1.8	8.3	2.7	1.0	1.5	5.5	1.1	33.0
Asp	27.4	3.1	9.3	7.1	1.7	6.2	8.2	2.1	65.1
Thr	17.0	2.2	5.7	4.0	1.7	1.5	4.2	1.4	37.7
Ser	18.9	1.9	8.2	5.9	1.2	1.5	4.8	0.4	42.8
Glu	30.4	3.4	16.3	0.6	3.2	6.7	6.7	2.0	69.3
Pro	10.5	1.3	5.7	2.0	0.8	1.1	2.3	1.1	24.8
Gly	16.4	6.6	12.1	3.8	1.9	1.8	3.4	5.9	51.9
Ala	25.5	5.6	7.9	5.8	2.2	2.8	1.2	4.9	55.9
Half-Cys	0	0	0	0	0	0	0	0	0
Val	20.2	3.1	8.2	5.4	1.2	4.2	6.1	4.6	53.0
Met	0	0	0	0	0	0	0	0	0
Ile	13.8	0.6	3.2	1.8	0.8	0.9	4.7	0.2	26.0
Leu	21.3	4.6	7.6	4.9	1.3	2.9	1.8	3.8	48.2
Tyr	9.8	1.3	3.6	2.5	0.2	0.2	0	1.7	19.4
Phe	11.4	0.9	5.9	3.4	0.8	1.6	1.1	0.0	25.1
Homoserine + homoserine lactone	1	1	1	1	0	1	1	1	7
Min mol wt[a]	31,169	4265	13,453	6577	2504	5173	8376	3174	74,691

[a] The minimum molecular weight for each polypeptide was estimated from amino acid composition data, assuming one residue of homoserine plus homoserine lactone per polypeptide. The exception was RCMCF2-III, whose minimum molecular weight was based on 1 mol of carboxymethylcysteine per mol.

rin and its CNBr fragments against anti-ovotransferrin showed that both CF1 and CF2 possess partial antigenic identity with ovotransferrin, which indicated that those regions of CF1 and CF2 containing antigenic determinants must reside on the surface of the intact molecule. In addition, CF1 and CF2 displayed complete antigenic identity to one another. CF3 and CF4 did not produce a precipitable product with anti-ovotransferrin. That they may still possess antigenic determinants must await further study.

Discussion

In certain respects, the results of the present investigation are reconcilable with previously reported observations on the CNBr fragments from ovotransferrin (Phillips and Azari, 1971). For example, on the basis of minimum molecular weight, CF1 and CF2 in the present report are identical with CB1 and CB2,while the mixture of CF3 and CF4 is similar to CB3-II. No incomplete cleavage product, identified previously as CB3-I, however, was produced in this study. On the other hand, there are certain important discrepancies between our present results and previous data of Phillips and Azari (1971), particularly with respect to the molecular weight of the fragments.

In the present investigation, values for the molecular weights of the fragments and their constituent polypeptides have been based primarily on composition analysis. Estimation of molecular weight was also attempted by several procedures, including sodium dodecyl sulfate–gel electrophoresis and gel filtration. However, anomalous behavior was noted for several of the fragments and polypeptides. For example, with Bio-Gel P-150 chromatography, in 1 M propionic acid, a

TABLE III: N-Terminal Amino Acids of the CNBr Fragments and Their Polypeptide Subunits.

Fragment[a] or Polypeptide	N Terminal[b]
CF1	Ala, Lys
RCMCF1-I	Ala
RCMCF1-II	Lys
CF2	Gly, Ala, Phe
RCMCF2-I	Gly
RCMCF2-II	Phe
RCMCF2-III	Ala
CF3	Asp
CF4	Thr, Glu
RCMCF4-I	Thr
RCMCF4-II	Glu

[a] The fragments (CF1, CF2, CF3, and CF4) are those obtained after CNBr cleavage of ovotransferrin. Polypeptides were obtained by reduction and carboxymethylation of the fragments. [b] N-Terminal amino acids were determined by reaction with 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) chloride. Dansyl amino acids were identified by tlc on polyamide plates in benzene–acetic acid (9:1, v/v). These results were confirmed by Edman degradation, followed by acid hydrolysis of the resulting phenylthiohydantoins.

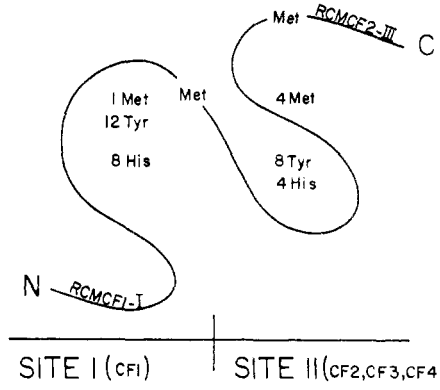

FIGURE 5: A tentative graphic representation of the primary structure of ovotransferrin. The molecule is arbitrarily divided into two halves. Site I is composed primarily of CF1, with its larger polypeptide chain, RCMCF1-I, placed at the N-terminal end of the intact protein. Site II represents the remaining CNBr fragments composed primarily of CF2, CF3, and CF4. With the exception of N- and C-terminal segments (RCMCF1-I and RCMCF2-III) the position of other segments in the primary structure is unknown. Because of this, only two methionines have been unequivocally positioned in the model.

reciprocal relationship was found between log molecular weight and the concentration of protein. This type of dependence of elution volume on protein concentration has been demonstrated in several studies (Nichol *et al.*, 1973). Also, the estimation of molecular weight by sodium dodecyl sulfate–gel electrophoresis was found to be useful only in the mol wt range of 80,000–13,000 daltons.

Another area of discrepancy between the present investigation and that of Phillips and Azari (1971) is with respect to the presence of additional polypeptide chains in each CNBr fragment. On the basis of eight methionines, CNBr cleavage of ovotransferrin should have theoretically produced nine fragments. In the present investigation, we were successful in further cleaving the CNBr fragments by reduction and carboxymethylation, producing a total of eight polypeptides. Furthermore, one residue of free homoserine plus homoserine lactone was identified. The latter finding indicates the presence of a single Met-Met sequence in the primary structure of ovotransferrin.

The total homoserine–homoserine lactone found in the present work accounts for a total of eight methionine residues in the native protein. Eight N-terminal amino acids were also found, of which alanine was found in two polypeptides. This finding is also in disagreement with previously reported N-terminal amino acids found for the uncleaved fragments. The present results indicate that ovotransferrin is composed of eight unique CNBr fragments, the largest of which represents half the molecular size of the protein. Similar results have been reported for the primary structure of human serum transferrin which showed the presence of a total of six unique fragments after CNBr cleavage (Bezkorovainy and Grohlich, 1972). However, no apparent homologies are seen between the CNBr fragments of ovotransferrin and human serum transferrin, particularly in respect to molecular weights and the constituent polypeptides.

The sequential order of the fragments is under further investigation. However, the positions of two of the polypeptides can be assigned from the present data. Since alanine was found to be the N-terminal amino acid of intact ovotransferrin (Fraenkel-Conrat and Porter, 1952), one of two polypeptides, RCMCF1-I or RCMCF2-III, qualifies for the N-terminal position in the intact protein. The absence of homoserine and homoserine lactone in RCMCF2-III indicates that the position of this polypeptide is at the C-terminal end; therefore the position of RCMCF1-I is assigned to the N-terminal end of the protein molecule.

A tentative graphic representation for the primary structure of ovotransferrin is presented in Figure 5. Several points of interest are depicted in the proposed model. (1) At least 5 of 7 methionines are located in the C-terminal half of the molecule (the one additional methionine attributable to the single Met-Met sequence in ovotransferrin is not shown in the diagram). (2) CF1 contains a significantly larger number of tyrosine and histidine (12 and 8 residues, respectively) as compared to the rest of the molecule (8 tyrosines and 4 histidines). In view of the involvement of these residues in metal-binding activity, their uneven distribution between the two halves of the molecule may be indicative of inequality of the two sites. (3) CF1 represents approximately half of the intact molecule. This fragment is now shown to contain one of the metal-binding sites (Tsao *et al.*, 1974). The second metal-binding site is apparently composed of the remaining three fragments (six polypeptides), none of which shows metal-binding activity individually.

These observations support the presently held view, based on epr studies, that the two metal-binding sites of ovotransferrin are dissimilar or unequivalent (Aasa, 1972). In conclusion we would like to emphasize that on the basis of CNBr fragmentation, the primary structure of ovotransferrin is not duplicated. However, there may still exist homologous regions of amino acid sequences in the two halves of the molecule. The eventual knowledge of the complete sequence of ovotransferrin will resolve this question.

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On the Structure of Ovotransferrin. II. Isolation and Characterization of a Specific Iron-Binding Fragment after Cyanogen Bromide Cleavage†

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ABSTRACT: Cleavage of ovotransferrin with cyanogen bromide at 4° resulted in the production of four fragments which were purified by Bio-Gel P-150 chromatography using 1 M propionic acid as the eluent. Iron-binding activity was found only in the largest fragment, denoted CF1 (mol wt 36,000), which consisted of two polypeptides linked by disulfide bonds. CF1 specifically bound 1 mol of Fe(III)/mol, and the iron complex exhibited maximum absorbance at 430 nm and a molar absorptivity coefficient approximately one-half that of saturated 2Fe-ovotransferrin. The binding of iron by apoCF1 could be destroyed by reduction and carboxymethylation of the fragment, denaturation by guanidine or urea, proteolysis, and iodination. Iron-CF1, on the other hand, exhibited enhanced resistance to these various treatments. This is the first time that an iron-binding fragment, representing only one of the iron-binding sites, has been isolated from ovotransferrin.

The transferrins are a group of homologous glycoproteins which are found in a variety of biological fluids, such as milk, plasma, and egg white (Feeney and Komatsu, 1966). These proteins possess very similar physical and chemical properties, and in particular, they show a strong affinity for the chelation of iron. They all have a molecular weight of about 80,000 daltons, and only a single polypeptide chain has been demonstrated for several of the transferrins after complete reduction and alkylation of all disulfide bonds in the presence of high concentrations of denaturing agents (Greene and Feeney, 1968; Bezkorovainy and Grohlich, 1967; Mann *et al.*, 1970). These proteins combine with ferric ion in a stoichiometric ratio of 2 atoms of Fe(III) to each protein molecule, producing a salmon-pink color with maximum absorbance at 470 nm (Feeney and Komatsu, 1966).

A variety of physical techniques, including electrometric and spectrophotometric titrations and electron paramagnetic resonance (epr), have implicated two–three tyrosines and two histidines in the chelation of each atom of iron by the protein (Windle *et al.*, 1963; Wishnia *et al.*, 1961; Aasa *et al.*, 1963; Tan and Woodworth, 1969). Additionally, spectroscopic data obtained from both proton magnetic resonance studies and the use of trivalent lanthanide ions as fluorescent probes have implicated the direct involvement of two tyrosines in each iron-binding site (Woodworth *et al.*, 1970; Luk, 1971). Recent work on the iodination of ovotransferrin (also known as conalbumin) has confirmed the involvement of two tyrosines and, in addition, the possible participation of one tryptophan residue in the binding of each iron atom (Phillips and Azari, 1972). Spectroscopic investigations of other workers have produced evidence which suggested that the two metal-binding sites of ovotransferrin may possess comparable binding strengths and thus the protein would bind metal ions at random (Aasa *et al.*, 1963).

Ovotransferrin is found in chicken egg white and has been the subject of structural investigations in this laboratory. Previous attempts to isolate a specific metal-binding fragment from ovotransferrin have been unsuccessful. This is because the iron complex of the protein was shown to be extremely resistant to proteolysis and to chemical and physical treatments, while apoovotransferrin,[1] on the other hand, could be hydrolyzed extensively, producing peptides which were devoid of specific metal-binding activity (Azari and Feeney, 1958, 1961). Recently, cyanogen bromide (CNBr) has been used for the specific cleavage of ovotransferrin at peptide bonds containing methionine (Tsao *et al.*, 1974), thus producing four fragments[2] (eight polypeptides) which were characterized with respect to composition and molecular weight. The iron-binding activity of the mixture of these CNBr fragments was tested and quite unexpectedly it was found that the mixture of fragments (after removal of CNBr and formic acid) produced a pink-orange color at pH 8.5 upon the addition of iron citrate. This indi-

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[1] Abbreviations used are: Fe-ovotransferrin, saturated iron complex of ovotransferrin, containing 2 mol of Fe(III)/mol of protein. Note that apoovotransferrin and apo-CF1 are equivalent to ovotransferrin and CF1, respectively; the prefix "apo" is used merely to emphasize the absence of metal.

[2] The designation of "fragments" and "polypeptides" as applied to the CNBr cleavage products of ovotransferrin has been adopted previously (Tsao *et al.*, 1974).