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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).

cated that some or all of the fragments had retained specific iron-binding activity. We, therefore, employed DEAE-cellulose chromatography to fractionate the mixture of CNBr fragments plus iron to determine which fragment or fragments contributed to the observed chromogenic activity. It was found that, in addition to the complete mixture of fragments, specific iron-binding activity resided only in the largest fragment, designated previously as CF1 (mol wt 36,000). This fragment is approximately one-half the size of native ovotransferrin, and apparently contains one of the iron-binding sites of the protein.

Experimental Procedure

Materials. Ovotransferrin was a 4× crystallized preparation (Azari and Baugh, 1967) and appeared homogeneous by acrylamide gel electrophoresis, in both acid and alkaline buffer systems containing urea, and by isoelectrofocusing.

Cyanogen bromide was purchased from Eastman Kodak (Organic Chemical Division). Propionic acid (buffer grade) was a product of Pierce Chemical Co. Sephadex G-25 (Superfine) was obtained from Pharmacia, and Dowex 1-X4 from Baker Chemical Co. Bio-Gel P-150 (100–200 mesh) was a product of Bio-Rad Laboratories. DEAE-cellulose (DE-32) was purchased from Whatman. "Ferrozine," used in the colorimetric determination of iron, was obtained from Hach Chemical Co. Iodine (Baker Analyzed Reagent) was resublimed before use. Spectrapor 3 membrane tubing (mol wt cut-off 3500 daltons) was purchased from Spectrum Medical Industries.

All other reagents were of the highest quality available and used without further purification.

Methods. CNBr CLEAVAGE OF OVOTRANSFERRIN AND SEPARATION OF FRAGMENTS. Approximately 100 mg of ovotransferrin was dissolved in 3 ml of ice-cold 70% formic acid, with the subsequent addition of 2 ml of ice-cold CNBr solution (1 g of CNBr/10 ml of 70% formic acid), to provide a 150-fold molar excess of CNBr over methionine content of the protein (assuming 8 mol of methionine/mol of ovotransferrin). The reaction was allowed to proceed 36 hr in a nitrogen atmosphere at 4° with gentle stirring. Two methods were then employed to prepare the CNBr fragments for chromatography. In one the CNBr reaction mixture was freeze-dried and the residue dissolved in 1 M propionic acid prior to gel filtration on Bio-Gel P-150. In the second, the reaction mixture was first dialyzed exhaustively against distilled water in Spectrapor 3 membrane tubing (mol wt cut-off 3500 daltons), followed by dialysis against 0.01 M NH_4HCO_3 (pH 8.3) for 48 hr. A white, alkaline-insoluble precipitate was formed in the dialysis bag. After centrifugation, the precipitate was dissolved in 3 M urea and its composition ascertained by acrylamide gel electrophoresis. It was found that the precipitate consisted mainly of ovotransferrin and small amounts of CF1 and CF2. The precipitate was subsequently discarded. The material remaining in solution (85% of the starting protein) was used for DEAE-cellulose chromatography, both with and without the addition of iron.

All fractionations were performed at 4°. Chromatography with Bio-Gel P-150 using 1 M propionic acid as the eluent was performed as previously described (Tsao *et al.*, 1974). DEAE-cellulose chromatography was performed as recommended by Roy and Konigsberg (1972). After precycling, removing fines, degassing, and equilibrating, the DEAE-cellulose was packed to a depth of 40 cm in a 2.5 × 60 cm column. The equilibrating buffer was 0.01 M NH_4HCO_3 (pH 8.3). The dialyzed reaction mixture, containing 200 mg of CNBr fragments at pH 8.3 and iron citrate (the amount of iron added was calculated to provide a 3× molar excess, based on a stoichiometry of Fe–ovotransferrin of 2:1), was applied to the column. Elution was performed with a linear salt gradient from 0 to 65 mM NaCl in 0.01 M NH_4HCO_3 (pH 8.3). Effluent fractions of 4 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. The chromogenic activity of each pooled fraction was tested by dissolving in 0.2 M NH_4HCO_3 (pH 8.5) and addition of iron citrate.

DETERMINATION OF IRON-BINDING CAPACITY. The iron-binding capacity of CF1 was determined by two procedures. In the first the amount of iron specifically bound to the protein was determined by a modified procedure employing "ferrozine" as a specific iron-binding agent (Stookey, 1970). First, CF1 was saturated with iron by the addition of iron citrate to a solution of CF1 in 0.1 M NH_4HCO_3 (pH 8.5). This solution was then applied to a short column of Dowex 1-X4 equilibrated with 0.1 M NH_4HCO_3 (pH 8.5) to remove the excess iron. The effluent was collected, dialyzed at pH 8.5, and freeze-dried. Preliminary experiments showed that the quantitative release of iron from FeCF1 could be achieved at acid pH. Additionally, a high concentration of denaturing agent was incorporated to keep the protein in solution. Consequently, the procedure used was as follows: to 0.05 ml of FeCF1 solution (0.5 mg of protein) was added 0.1 ml of 7 M guanidine·HCl. This was followed by the addition of 0.5 ml of "ferrozine" solution (composition: 257 mg of ferrozine, 5 g of NH_2OH–HCl, 25 ml of concentrated HCl, and water to a final volume of 50 ml). After 10 min at room temperature, 2.35 ml of ammonium acetate–urea solution was added (composition: 20 g of ammonium acetate dissolved in 4 M urea, pH adjusted to 9.5 with concentrated NH_4OH, and diluted to a final volume of 50 ml with water). The mixture was allowed to stand at room temperature for 30 min and the absorbance of the solution was read at 562 nm against a proper blank. The amount of iron was determined from a standard curve. Controls were prepared with protein added (ovotransferrin or CF1) to correct for any nonspecific iron binding or incomplete release of iron.

In the second procedure CF1 was dissolved in 0.1 M citrate (pH 4.7) and passed over a small column of Dowex 1-X4 equilibrated with the same buffer in order to remove the protein-bound iron. Column effluent was dialyzed exhaustively against distilled water and freeze-dried. The iron-binding capacity of the apo-CF1 was then determined by measuring the increase in absorbance at 430 nm upon the addition of small increments of iron citrate solution.

Gel electrophoresis and reduction–carboxymethylation were performed as described (Tsao *et al.*, 1974). To remove reagents and salts from the carboxymethylated protein, the reaction mixture was passed through a 1 × 100 cm column of Sephadex G-25 equilibrated with 0.1 M NH_4HCO_3 (pH 8.0) and then freeze-dried.

TRYPSIN HYDROLYSIS OF CF1 AND FeCF1. A 1% protein solution was prepared by dissolving apo-CF1 or iron-saturated CF1 in 0.1 M NH_4HCO_3 (pH 8.5). L-1-Tosylamido-2-phenylethyl chloromethyl ketone treated trypsin, in an amount equal to 2% of protein by weight, was added and the solution stirred for varying periods at 25°. The residual chromogenic activity of FeCF1 was determined directly from the absorbance of the solution at 430 nm. In the case of apo-CF1 sufficient iron citrate was added to form the saturated complex. The colored complex was developed for 5 min and the absorbance determined at 430 nm.

IODINATION. Short-time iodination of CF1 and FeCF1 at different iodine concentrations was conducted as described by

Phillips and Azari (1972). In this study, 4 mg of protein (apo-CF1 or iron-saturated CF1) was dissolved in 0.8 ml of 0.1 M $NaHCO_3$ (pH 9.0). The solution was chilled at 0° for 30 min and to it was added with agitation 5 μl of chilled iodine solution (0.05 M I_2–0.1 M KI). The solution was incubated (with agitation) for 1 min. Reaction was then terminated by adding 50 mM arsenite solution equivalent to the iodine used. The solution was warmed to room temperature and the final volume was made up to 1.0 ml with buffer. This procedure was repeated at different concentrations of iodine up to a maximum of 5.6 mM.

The molar ratio of iodine to protein tyrosine ranged from 0.25 to 4, based on 12 mol of tyrosine/mol of CF1. The residual chromogenic activity of IFeCF1 was determined directly from the absorbance of the solution at 430 nm. In the case of ICF1, sufficient iron citrate solution was added to the mixture to saturate the protein. The colored complex was developed for 15 min and the absorbance was determined at 430 nm against a suitable blank. The extent of conversion of tyrosine to diiodotyrosine was determined spectrophotometrically from the difference in absorbance between modified and unmodified preparations. To this end, 0.2 ml of reaction mixture was diluted to 2 ml with 0.2 M glycine–NaOH (pH 10.5) and the moles of diiodotyrosine per mole of the modified proteins were calculated from the difference in the absorbance at 314 nm for ICF1 *vs.* CF1 control and for IFeCF1 *vs.* FeCF1 control. A molar extinction coefficient of 5.55×10^3 for diiodotyrosine and a mol wt of 40,000 for CF1 were employed for these calculations.

Results

CNBr cleavage of ovotransferrin at 4° resulted in the production of four fragments: CF1, CF2, CF3, and CF4 which were separated and purified, as previously described in detail (Tsao *et al.*, 1974). Cleavage with CNBr at 4° produced alkaline-soluble fragments which could be tested easily for iron-binding activity, especially the ability to produce a colored complex. CF1 was the only fragment which produced a yellow-orange colored complex upon the addition of iron. However, in several preliminary experiments we have observed that fractionation of the CNBr fragments in 1 M propionic acid resulted in a gradual decrease in the iron-binding activity of CF1. This was also usually accompanied by a significant decrease in the solubility of the fragment at alkaline pH. The chromatographic separation of the fragments on DEAE-cellulose was found to avoid these problems, and, in addition, provided a means of detecting and isolating any fragment(s) possessing chromogenic activity. A typical elution profile for DEAE chromatography of the CNBr digest is shown in Figure 1. In this instance, the solution of the CNBr fragments also contained excess iron citrate. Hence, the colored iron–protein complexes could be located easily in the column effluent. In this way, color was observed in fractions V (pink) and in VI and VII (yellow-orange). The indicated regions of Figure 1 were pooled into seven fractions, dialyzed against distilled water in Spectrapor 3 membrane tubing (mol wt cut-off 3500), and then freeze-dried. The homogeneity of each fraction was tested by acid–urea gel electrophoresis. The electrophoretic pattern of these fractions is shown in the insert of Figure 1. The identity of each CNBr fragment in each fraction is indicated as CF1, CF2, CF3, and CF4, the same as reported previously (Tsao *et al.*, 1974). It is evident that a total recovery of individual fragments by the DEAE procedure is impractical; however, fractions I, II, VI, and VII show predominantly one

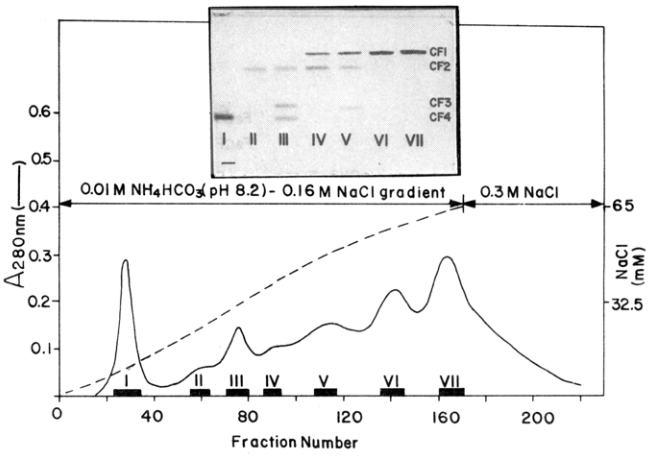

FIGURE 1: DEAE-cellulose chromatography of CNBr-cleaved ovotransferrin. The CNBr fragments of ovotransferrin were prepared as described under Methods. The dialyzed reaction mixture, containing 200 mg of CNBr fragments at pH 8.3 plus excess iron citrate, was applied to a 2.5 × 60 cm column of DEAE-cellulose (DE-32). Elution was performed with a linear salt gradient from 0 to 65 mM NaCl in 0.01 M NH_4HCO_3 (pH 8.3). Fractions of 4 ml were collected. Horizontal bars indicate the fractions pooled. The insert shows the acrylamide gel electrophoresis pattern of each pooled fraction. Conditions for electrophoresis were those described previously (Phillips and Azari, 1972).

band. This was especially advantageous in the case of fraction VII which contained a high concentration of CF1 and could be used directly without further purification.

We have also attempted DEAE-cellulose chromatography of the CNBr fragments in the absence of iron. The elution pattern was similar to the one shown in Figure 1; however, resolution of protein peaks was increased. It appears that the presence of iron causes a more extensive association of CNBr fragments. This was further supported by sedimentation analysis of the complete mixture of CNBr fragments in the presence and absence of iron. The fastest sedimenting component in the iron-free solution of fragments corresponded to CF1 (mol wt 36,000), while the solution of fragments containing iron showed a quickly sedimenting component corresponding to a mol wt of about 60,000. We have been particularly interested in testing the specific iron-binding activity of intact CF1, because it represents about one-half of the ovotransferrin molecule, and apparently contains one of the metal-binding sites. The chemical composition, molecular weight, and the polypeptide components of CF1 have been described previously (Tsao *et al.*, 1974).

The visible absorbance spectra for FeCF1, the complete mixture of CNBr fragments in the presence of iron, and the iron-saturated ovotransferrin are shown in Figure 2. A broad absorbance band with a maximum at 430 nm is observed for FeCF1, which is significantly lower than 460–470 nm observed for the mixture of fragments, as well as for Fe–ovotransferrin. It must be pointed out that although the maximum absorbance for the mixture of fragments appears at 460 nm, which is very close to that of native Fe–ovotransferrin, no uncleaved ovotransferrin was present in the CNBr-treated mixture. Hence, the mixture of CNBr fragments apparently is capable of producing near-native iron-binding activity. The molar absorptivity coefficients for these preparations are shown in the insert of Figure 2. The ϵ value for CF1 is approximately one-half that of Fe–ovotransferrin, whereas that of CNBr–ovotransferrin fragments is close to that of Fe–

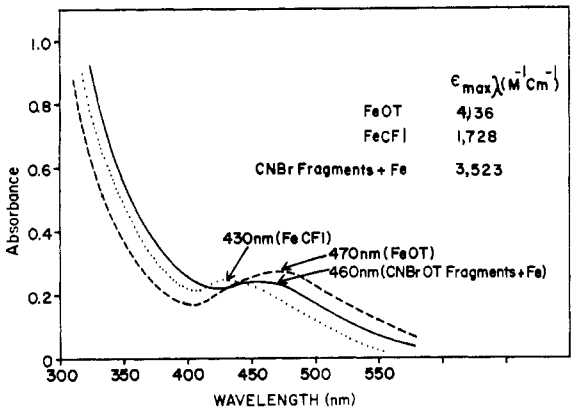

FIGURE 2: Visible absorbance spectra for FeCF1 (· · ·), Fe–ovotransferrin (---), and the mixture of CNBr fragments in the presence of iron (—). Proteins were dissolved in 0.2 M NH_4HCO_3 (pH 8.5) at a concentration of 5 mg/ml. The molar absorptivity coefficients for these preparations are shown in the insert and were calculated on the basis of mol wt of 36,000 for CF1 and 76,600 for Fe–ovotransferrin as well as CNBr fragments.

TABLE I: Iron-Binding Capacity of CF1 by the "Ferrozine" Procedure.

Protein[a]	mmol of Fe/mg of Protein	mol of Fe/mol of Protein[b]
Fe–ovotransferrin	24.8	1.96
Fe–ovotransferrin[c]	24.8	1.96
FeCF1[d]		
1	24.0	0.96
2	24.5	0.98
3	24.0	0.96

[a] Iron-saturated proteins were prepared as described under Methods; 0.5 mg of iron-saturated protein was used for each determination. [b] Calculated on the basis of mol wt of 80,000 for Fe–ovotransferrin and 40,000 for FeCF1. [c] The ovotransferrin in this preparation was treated under the same conditions employed for CNBr cleavage, only omitting CNBr. [d] The numbers denote different preparations of CF1.

ovotransferrin. The ϵ value for the 70% formic acid treated ovotransferrin was the same as for the untreated preparation.

The iron-binding capacity of intact CF1 was determined by spectrophotometric titration with iron as shown in Figure 3. From the intercept of the two lines, a value of 0.98 mol of iron/mol of CF1 was calculated. It should be noted that the addition of iron citrate to apo-CF1 during titration resulted in an increase in visible absorbance only at 430 nm. Table I shows the results of determining the iron-binding capacity of FeCF1 directly from the amount of iron specifically bound by the protein. Saturated Fe–ovotransferrin was used as a control in order to check the reliability of the ferrozine procedure. Quantitative recovery of about 2 mol of iron per mol of ovotransferrin indicates that the method is reliable. It is seen that several preparations of FeCF1 gave a stoichiometry of about 1 mol of iron/mol of CF1. Other CNBr fragments showed only negligible iron-binding activity.

The specific chromogenic and iron-binding activity of CF1 could be destroyed by various physical and chemical treatments, including reduction and carboxymethylation, denaturation in 6 M urea or 4 M guanidine·HCl, heating at 60°, and proteolysis. The rate of loss of chromogenic activity of FeCF1 and apo-CF1 in the presence of trypsin is shown in Figure 4. The chromogenic activity is expressed as the residual absorbance of FeCF1 (directly) or apo-CF1 (after addition of iron citrate) at 430 nm. It is seen that FeCF1 is significantly more resistant to tryptic hydrolysis than apo-CF1. In 30 min apo-CF1 shows a 90% loss of chromogenic activity as compared to only a 20% loss for FeCF1 for the same time period.

The chromogenic activity of CF1 could also be destroyed by iodination of tyrosine residues. Figure 5 shows the extent of loss of chromogenic activity for FeCF1 and apo-CF1 at different iodine concentrations. It is seen that FeCF1 retains 90% of its chromogenic activity after reaction with 0.6–5.6 mM iodine. In contrast, there is a gradual decrease in the chromogenic activity of apo-CF1, with a maximum loss of 70% at 5.6 mM iodine. The iodinated apo-CF1 (70% loss of chromogenic activity) showed a modification of 4 tyrosines (out of 12). Approximately the same number of modified tyrosines was also found for iodinated FeCF1 (showing 10% loss of chromogenic activity).

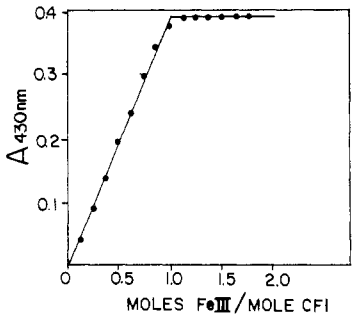

FIGURE 3: Spectrophotometric titration of CF1 with iron citrate. CF1 was dissolved in 0.2 M NH_4HCO_3 at a concentration of 4 mg/ml. The increase in absorbance at 430 nm was determined after the addition of increments of standard iron citrate solution (20 μmol of Fe(III)/ml).

Discussion

To our knowledge this is the first time that a specific iron-binding fragment from ovotransferrin has been isolated. This achievement was predominantly due to the CNBr cleavage of

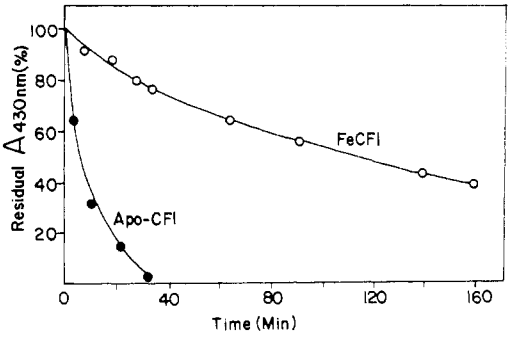

FIGURE 4: Time course of the tryptic hydrolysis of CF1 and FeCF1. Protein concentration was 1% in 0.1 M NH_4HCO_3 (pH 8.5). L-1-Tosylamido-2-phenylethyl chromethyl ketone treated trypsin added represented 2% the weight of the protein. Incubation temperature was 25°. The residual chromogenic activity of the protein was determined from the absorbance at 430 nm, directly for FeCF1 and after the addition of excess iron citrate for apo-CF1.

ovotransferrin at low temperature, which produced alkaline-soluble CNBr fragments. A further important step in the isolation of the pure iron-binding CF1 fragment, in good yield, was the use of DEAE-cellulose chromatography.

In this investigation we have been particularly interested in the specific iron-binding activity of the largest CNBr fragment designated as CF1. The factors responsible for the co-elution of different fragments from DEAE-cellulose are not clear at present. The presence of iron appears to be specific because several of the possible combination of fragments (such as CF1 and CF3) are absent from the electrophoretic pattern. It is also possible that a prolonged exposure of fragments to acid conditions (during CNBr cleavage) may cause sufficient change in the conformation and the charge of the fragments, producing various molecular species. This may be the case with fractions VI and VII, both of which show chromogenic activity and are substantially pure, but elute at different conditions.

While these phenomena are under further investigation, several conclusions can be tentatively drawn as to the contribution of the fragments to the chromogenic activity of ovotransferrin. For example, since fraction V is composed of CF1, CF2, and CF3, and also shows a pink coloration similar to native Fe–ovotransferrin, it may be concluded that CF4 does not contribute to the chromogenic activity of ovotransferrin. On the other hand FeCF1 devoid of other fragments is capable of binding iron; however, the significant difference of its maximum absorbance (430 nm) as compared to Fe–ovotransferrin (470 nm) indicates that the iron–protein chelate structure of this fragment must be quite different than that of native Fe–ovotransferrin. Also, the maximum absorbance and the molar absorptivity of the mixture of all fragments plus Fe are found to be very close to that of native Fe–ovotransferrin. It therefore appears that the mixture of fragments can assume the conformation of the two metal-binding sites of the native molecule. A more precise contribution of each fragment to the total chromogenic activity of the native Fe–ovotransferrin is under further investigation and will be reported in a separate communication.

The mol wt of about 36,000 daltons found for CF1 and its stoichiometric binding of 1 mol of iron/mol indicate that this fragment is derived from approximately one-half of the ovotransferrin molecule and must contain one of the iron-binding sites of native protein. This is further supported by the spectral data for saturated FeCF1 which indicate a molar absorptivity value of approximately one-half that of saturated 2Fe–ovotransferrin.

The specificity of the iron binding by CF1 was tested by several procedures. For instance, the ability of apo-CF1 to bind iron was destroyed by reduction and carboxymethylation, denaturation in guanidine or urea, heating, proteolysis, and iodination. Also, in contrast to Fe–ovotransferrin, which has been shown to be extremely resistant to proteolysis as compared to apo–ovotransferin (Azari and Feeney, 1958), FeCF1 showed a gradual decrease in chromogenic activity, although at a much slower rate than apo-CF1. Iodination of apo-CF1 resulted in an extensive loss of chromogenic activity, whereas FeCF1 retained nearly full activity under similar conditions. These observations are analogous to the results reported for the iodination of ovotransferrin and Fe–ovotransferrin (Phillips and Azari, 1972). The extent of tyrosine modification was found to be the same for both FeCF1 and apo-CF1 (4 mol of diiodotyrosine/mol of protein). The interpretation of these results must await a more detailed investigation. At present not much is known about the geometry of the two metal-binding sites. However, since CF1 has been previously assigned to the N-terminal region of the ovotransferrin molecule (Tsao *et al.*, 1974), the position of one of the metal-binding sites must be in this region. The second iron-binding site of ovotransferrin presumably would be contained within the remaining CNBr fragments. On the basis of the present investigation it would appear that the gross primary structure around the two iron-binding sites in ovotransferrin must be dissimilar.

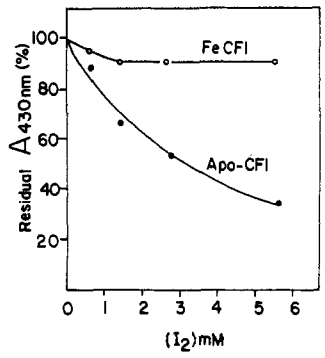

FIGURE 5: Iodination of apo-CF1 and FeCF1 at different iodine concentrations. The reaction mixture contained 5 mg of protein/ml in 0.1 M $NaHCO_3$ (pH 9.0). The reaction was conducted at 0° for 1 min. The chromogenic activity is represented as the residual absorbance of iron-saturated protein at 430 nm. This was determined directly for iodinated FeCF1 and after addition of excess iron citrate to iodinated apo-CF1.

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