densed, reactions A and B, it appeared that reaction B (electrophilic attack at C-2) was the preferred one. This was shown in Figure 2 and 3 which indicate that pyrrole consumption and porphyrin formation were considerably higher at neutral pH than at pH 3.5. Since reaction mechanism B will be strongly inhibited at pH 3.5, we conclude that this mechanism is the preferred for porphobilinogen polymerization. Reaction mechanism A (electrophilic attack at C-5) is preferred only when the electron-releasing properties of the substituent at C-4 increase. By replacing the propionic acid residue by a methyl group, as in MPMA, pyrrole consumption rates and porphyrin formation rates became higher at pH 3.5 and were independent of activation by formaldehyde or inhibition by dimedon (Table I). Thus MPMA reacted almost exclusively by mechanism A. When the propionic acid residue was replaced by hydrogen, the resulting pyrrole (PMA) was very slow to self-condense and it almost did not yield porphyrins, thus indicating that the Mannich base is in this sense a nonreactive compound.

Porphobilinogen appears to be the "best choice" among the examined 2-aminomethylpyrroles. MPMA, which is more reactive, is very unstable and it starts to form porphyrins already at 37° (Figure 4). The uroporphyrins are the most stable among the homologous porphyrins. The porphyrins originated in MPMA are acid labile and the porphyrins originated PDA are unstable in the air. Porphobilinogen was the only pyrrole, among the examined, to form stable intermediates during the condensation process, which were transformed with time into porphyrinogens.

When the chemical condensation of porphobilinogen was compared with the enzymatic one, there was a striking difference. In the enzymatic reaction the yields were approximately 100%, even when measured at the possible shortest times (minutes). When the yields were lower than 100% (Frydman and Frydman, 1970), they did not increase with time. This was also the case when the enzymatic reaction was carried out at very low concentrations (1×10^{-5} M) of porphobilinogen. The enzymatic condensation is certainly an anaerobic process, but occurs also in the air giving almost the same porphyrin yields (Frydman and Frydman, 1970). We already mentioned in Results that this was not case when the uroporphyrins were formed chemically. It is thus apparent that the enzymatic polymerization of porphobilinogen takes place on the enzymatic surface without accumulation of free intermediates, at least under normal conditions.

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On the Structure of Ovotransferrin. Isolation and Characterization of the Cyanogen Bromide Fragments and Evidence for a Duplicate Structure*

Jerry L. Phillips and P. Azari†

ABSTRACT: The reaction of cyanogen bromide with ovotransferrin produced three polypeptide fragments, which were recovered in a mole ratio of 1:1:1. For every mole of ovotransferrin cleaved and fractionated, 2 moles of each fragment was recovered. Molecular weights of the fragments were estimated as 21,000, 9400, and 7000, the sum of which is approximately one-half that of the native protein (76,600). The amino acid and carbohydrate composition of the fragments was one-half that of the native protein. These results are consistent with a protein structure in which duplicate polypeptide fragments are linked to form a single polypeptide chain.

The transferrins are a group of homologous glycoproteins which bind two atoms of ferric ions in two separate but equivalent sites on the protein molecule (Warner and Weber, 1953; Warner, 1959; Aasa *et al.*, 1963; Windle *et al.*, 1963; Aisen *et al.*, 1966; Feeney and Komatsu, 1966). Because of their high molecular weights (*ca.* 80,000), it has been sug-

* From the Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80521. *Received August 5, 1970.* This investigation was supported by Research Grants GM 12029 from the National Institutes of Health, United States Public Health Service, and GB-7242 from the National Science Foundation. The material was taken from a thesis submitted by J. L. Phillips (Recipient of NSF Traineeship Award, 1968–1970) to the Graduate College of Colorado State University in partial fulfillment for the degree of Doctor of Philosophy in Biochemistry.

† To whom correspondence should be addressed.

gested that transferrins may possess a subunit structure (a dimer). Jeppsson (1967) has reported that human transferrin exists as a dimer with the monomer possessing a molecular weight 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 conclusively demonstrated that transferrins are not dimeric but consist of a single polypeptide chain. The possibility of duplicate internal structure has also been suggested from a variety of evidences. The electron spin resonance measurements indicated the presence of two equivalent metal binding sites (Windle *et al.*, 1963; Aisen *et al.*, 1966). A more direct form of evidence, however, is that of Jamieson (1965) who identified two identical carbohydrate side chains on human serum transferrin. Also, human serum transferrin, rabbit serum transferrin, and chicken serum transferrin all have been reported to yield fewer tryptic peptides than would be expected on the basis of total lysine and arginine content (Jeppsson, 1967; Baker *et al.*, 1963; Williams, 1962).

We have examined the possibility of repeating polypeptide segments in hen's egg white ovotransferrin by cleaving the protein at methionine residues with cyanogen bromide. The polypeptide fragments thus obtained were characterized by their molecular weights and by amino acid and carbohydrate content. These results indicated that ovotransferrin is a monomeric, single-chain protein possessing duplicate polypeptide segments.

Methods

Materials. The OT[1] used in this study was a seven-times-crystallized preparation, as described previously (Azari and Baugh, 1967). The protein appeared homogeneous by sedimentation velocity and by acrylamide gel electrophoresis.

Bio-Gel P-150 (100–200 mesh) was obtained from Bio-Rad Laboratories. Cyanogen bromide was an Eastman Kodak reagent. Propionic acid (buffer grade) was the product of Pierce Chemical Co. Homoserine lactone was synthesized by reacting L-methionine with an excess of cyanogen bromide. After a reaction time of 24 hr, the reaction was found to be 86% complete by amino acid analysis. Standard homoserine, α-chymotrypsin, cytochrome *c*, 5-dimethylamino-1-naphthalenesulfonyl (dansyl) chloride, and suitable dansylamino acid standards were all purchased from Sigma Chemical Co. Insulin was obtained from Squibb. Precoated silica gel thin-layer plates (6060) were a product of Eastman Kodak. All other reagents were of the highest available grade, and used without further purification.

CNBr Cleavage. The reaction of ovotransferrin with CNBr was conducted essentially as described by Gross (1967). Approximately 80-mg (1 μmole) samples of OT were dissolved in 6.9 ml of 70% formic acid. To this was added 1.1 ml of CNBr solution (1 g/10 ml of 70% formic acid), providing a 100-fold molar excess of reagent over methionine content. The reaction was allowed to proceed 5 hr in a nitrogen atmosphere at room temperature. To remove excess reagent and by-products, the reaction mixture was diluted ten times with distilled water and lyophilized. Lyophilization was repeated several times to ensure complete removal of volatile material.

Chromatography of CNBr Digest. Bio-Gel P-150 was allowed to swell at least 24 hr in 1 M propionic acid, and then packed to a depth of 110 cm in a 1.8 × 120 cm column. The column was equilibrated with 1 M propionic acid for 48 hr at a constant flow rate of 15 ml/hr. Samples of CNBr-treated OT (80 mg) were dissolved in 3–4 ml of 1 M propionic acid and applied to the column. Effluent fractions of 5 ml were collected, and the optical density of each was measured at 280 mμ in a Coleman 111 spectrophotometer. Appropriate fractions were pooled and lyophilized. Further purification of pooled fractions was accomplished by rechromatography on the same column of Bio-Gel P-150.

Acrylamide Gel Electrophoresis. Vertical slab electrophoresis was performed in the apparatus of E-C Corp. The discontinuous acid gel system of Jordan and Raymond (1969) was used, except that the concentration of acrylamide in the gels was 7%. The gel slabs were stained with amido black and then destained electrophoretically.

Amino Acid Analysis. Samples were hydrolyzed in glass-distilled 6 N HCl at 107° for 24 hr in sealed, evacuated tubes. The acid was removed by lyophilization after diluting the hydrolysate with distilled water. Analyses were performed as described by Spackman *et al.* (1958) on a Beckman 120B amino acid analyzer operated under accelerated conditions. No corrections were made for possible losses of serine, threonine, or tyrosine. The yield of cleavage at methionine residues was determined from the total homoserine plus homoserine lactone content.

Carbohydrate Analysis. Total hexose and hexosamine were determined as described previously (Azari and Phillips, 1970), using mannose and glucosamine, respectively, as standards.

N-Terminal Determinations. N-terminal amino acids of the fragments were determined by the cyanate procedure of Stark (1967). After hydrolysis of the resulting hydantoins the free amino acids were determined by amino acid analysis. Confirmation of the cyanate data was done by dansylation according to Bustin and Cole (1969). Dansylamino acids were identified by thin-layer chromatography on silica plates in the solvent system benzene–pyridine–acetic acid (80:20:2) by comparison with standard dansylamino acids. In cases where the identification by the above procedure was difficult, Edman degradative procedure was employed and the resulting phenythiohydantoin amino acids were identified by gas chromatography as described by Pisano and Bronzert (1969).

Reduction and Carboxymethylation of the CNBr Fragments. Reduction and carboxymethylation of the CNBr fragments were conducted by a modification of the procedure of Crestfield *et al.* (1963) Each fragment was separately dissolved in 0.3 M Tris-HCl (pH 8.6) containing 8 M urea to a final concentration of 0.5% (w/v). Dithioerythritol was added to provide a fivefold molar excess over disulfide content. Solutions were incubated in a nitrogen atmosphere for 4 hr at 33°. Iodoacetic acid, dissolved in 1 M NaOH (to give 2 moles of iodoacetic acid/1 mole of NaOH), was then added, and each solution was kept in the dark at room temperature for 20 min. After this time, the nitroprusside test for thiol groups was negative. The solutions were finally passed through a short column of Bio-Gel P-4 equilibrated with 1 M propionic acid and lyophilized.

Molecular Weight Determinations. Molecular weights were estimated from the amino acid composition of each fragment, assuming one residue of homoserine lactone for each fragment. Results thus obtained were confirmed by gel filtration studies on a 1.8 × 110 cm column of Bio-Gel P-150 with 1 M propionic acid as the eluent. Native OT was used to determine the void volume, while α-chymotrypsin (mol wt 22,500), cytochrome *c* (mol wt 12,500), and insulin (mol wt 5700) were used as standards. The molecular weights of the fragments

[1] Abbreviation used is: OT, ovotransferrin also known as conalbumin.

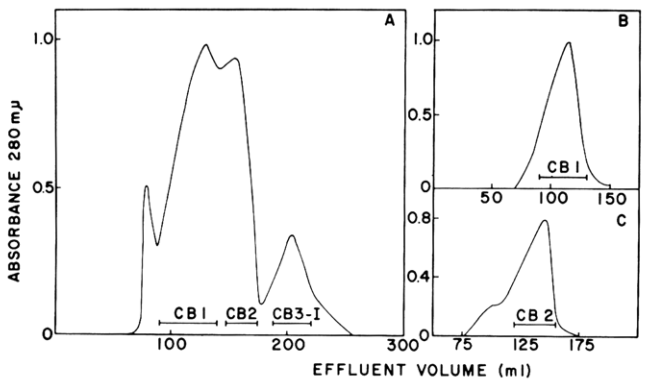

FIGURE 1: Fractionation of CNBr-treated OT on Bio-Gel P-150 is illustrated in Figure A. The eluent was 1 M propionic acid. Fractions of 5 ml were collected at a flow rate of 15 ml/hr. Horizontal bars indicate fractions pooled. Rechromatography of fragments CB1 and CB2 is illustrated in Figures B and C, respectively.

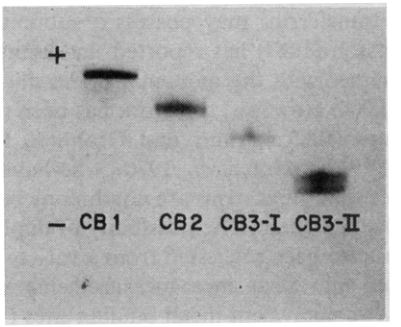

FIGURE 2: Acrylamide gel electrophoresis pattern of the purified CNBr fragments from OT. The gel composition was 7% cyanogum-41, 0.12 M Tris-citrate buffer, pH 2.9, and 3 M in urea. The electrode buffer was 0.37 M glycine–citrate, pH 4. Runs were made at 350 V (120 MA) for 2 hr at 1°. Each sample was approximately 50–75 μg of protein. Protein bands were stained with amido black and destained electrophoretically.

were estimated from a graph showing the logarithmic relationship of molecular weights to V_E/V_0. Membrane osmometry was performed according to the procedure of Prather *et al.* (1968) and was limited to the determination of high molecular weight fragments.

Results

CNBr Cleavage. Amino acid analysis showed that the conversion of methionine into hemoserine and homoserine lactone was approximately 96% complete after 5 hr. Extending the time of reaction to 24 hr did not result in additional conversion (or disappearance) of methionine.

Bio-Gel Chromatography of the CNBr Fragments. The elution pattern obtained by fractionation of the CNBr fragments from OT is presented in Figure 1A. The initial component eluted was identified by amino acid analysis as uncleaved OT, and amounted to about 4% of the total protein applied to the column. The next two components, CB1 and CB2, were incompletely resolved and required further purification by rechromatography, as seen in Figures 1B and 1C. The final component, CB3-I, was sufficiently resolved from CB2 and was not subjected to further purification. The yield for each resolved fragment was: CB1, 54%; CB2, 23%; and CB3-I, 19% (total 96%).

Each fragment was tested for homogeneity by acrylamide gel electrophoresis at pH values ranging from 2.6 to 8.9, and in the presence of 3 M urea or 0.1% sodium dodecyl sulfate.

A typical electrophoretic pattern is shown in Figure 2. The CB1, CB2 and CB3-I bands correspond, respectively, to the individual peaks in Figure 1. The CB3-II pattern of the two closely migrating bands was produced in most preparations of CNBr-treated OT by the procedure outlined above. However in a few preparations, even though exactly the same procedure was followed, we obtained an uncleaved fragment that we have designated as CB3-I.

The CB3-II fragments were eluted as a single symmetrical peak at a slightly larger volume than that of CB3-I shown in Figure 1. We were unsuccessful in separating the CB3-II components further by conventional chromatographic procedures. The amino acid composition of CB3-II was the same as for CB3-I shown in Table I (with the exception of methionine and homoserine lactone, see Discussion). The CB3-II showed an average molecular weight of approximately half that of CB3-I (4000 *vs.* 7000). On the basis of these data we concluded that CB3-II must be the complete cleavage product of CB3-I. For the sake of simplicity and more meaningful interpretation, further experiments were conducted predominantly on the uncleaved (homogeneous) CB3-I fragment.

Amino Acid and Carbohydrate Composition of the CNBr Fragments. Analyses of the CNBr fragments are presented in Table I. Compositions were calculated by mole ratios assuming one residue of homoserine lactone per fragment. The composition of native OT was computed based on a molecular weight of 76,600.

It must be noted that in spite of the asymmetric nature of the elution profiles for CB1 and CB2 (as seen in Figures 1B and 1C) the amino acid analyses showed a constant composition across the respective peaks for each fragment. With respect to the CB3-I, we found that it contained one residue of homoserine lactone and one intact methionine.

It is seen that the amino acid compositions and molecular weights of the three fragments account for approximately one-half of the total number of amino acid residues (324 *vs.* 651), as well as one-half of the molecular weight of the intact protein (37,400 *vs.* 76,600). Carbohydrate analyses for the three fragments are also presented in Table I and show that fragments CB1 and CB2 contain both mannose and glucosamine, while CB3-I is devoid of carbohydrate. Although mannose is present to the same extent on both fragments, CB2 contains twice as much glucosamine as CB1. It is also seen that the total carbohydrate content of the fragments is, like the amino acid composition, one-half that of native OT.

Molecular Weights of the CNBr Fragments. Figure 3 graphically presents the results of gel filtration studies. A linear relationship is seen to exist between the logarithm of the molecular weight of the three proteins investigated and the ratio of elution volume to void volume (V_E/V_0). The apparent molecular weights of the fragments were estimated from their V_E/V_0 values as indicated on the graph. These data are summarized in Table II along with the molecular weight values estimated by composition analysis and osmotic pressure. The molecular weights determined by these procedures are seen to compare favorably. As was mentioned previously, the sum of molecular weights for the three fragments total one-half the molecular weight of the native protein.

Stoichiometry and Recovery of the CNBr Fragments. The three CNBr fragments were found to exhibit similar extinction coefficients of 4500 M^{-1} cm^{-1} at 280 nm and at alkaline pH. This is also reflected in the tyrosine and tryptophan con-

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

Amino Acid	Residues/Molecule				
	CB1	CB2	CB3-I	Total	Native OT
Lysine	17	6	6	29	56
Histidine	4	1	Trace	5	12
Arginine	7	3	4	14	28
Aspartic acid	19	9	9	37	72
Threonine	11	5	3	19	33
Serine	12	6	3	21	40
Glutamic acid	18	8	8	34	67
Proline	9	3	2	14	26
Glycine	15	7	5	27	52
Alanine	18	6	4	28	55
Half-cystine	8	4	4	16	31
Valine	13	5	8	26	52
Methionine	0	0	1	1	8
Isoleucine	8	2	2	12	24
Leucine	14	6	5	25	49
Tyrosine	6	3	1	10	20
Phenylalanine	7	4	2	13	26
Homoserine lactone plus homoserine	1	1	1	3	0
Tryptophan[a]	3	1	1	5	12
Minimum mol wt[b]	21,000	9400	7000	37,400	76,600
Mannose[c]	1	1	0	2	4
Glucosamine[c]	1	2	0	3	6

[a] Tryptophan was determined colorimetrically with *p*-dimethylaminobenzaldehyde (Azari and Phillips, 1970). The number of residues per molecule was based on the minimum molecular weight per each fragment as shown in the table. [b] Minimum molecular weight for fragments was estimated from amino acid composition data, assuming one residue of homoserine lactone for each fragment. The molecular weight of OT is based on sedimentation data. [c] The number of residues were calculated on the basis of the molecular weight for each fragment and native OT as shown in the table.

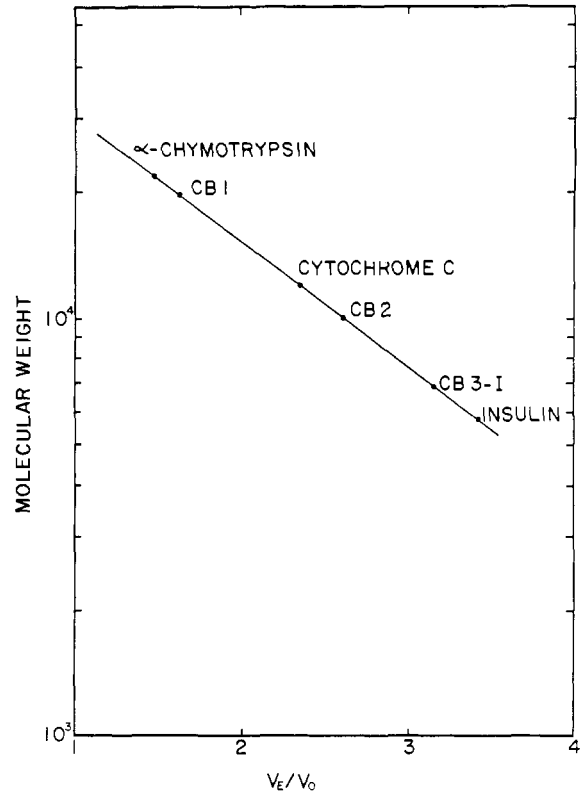

FIGURE 3: Molecular weight determination of the CNBr fragments by gel filtration. A 1.8 × 110 cm column of Bio-Gel P-150 was used, with 1 M propionic acid as the eluent. Fractions of 5 ml were collected at a flow rate of 15 ml/hr.

tent for each fragment as shown in Table I. Each fragment shows approximately the same number of tyrosine and tryptophan residues, respectively, per unit molecular weight (the exception is the tyrosine content of CB3-I which shows a relatively lower value). On this basis the area under the peak for each fragment (fro n Figure 1) should show a constant relation to the molecular weights, if the fragments are present in equimolar quantities. Table III shows the results of such a comparison, and indicates that the three fragments are recovered in a ratio of 1 : 1 : 1.

The molar quantity of each fragment was calculated from the actual yield for each purified fragment. In a typical experiment, for every 80 mg (1 μmole) of OT cleaved and fractionated, we obtained, as purified and homogeneous preparation, 40.9 mg of CB1, 17.6 mg of CB2, and 14.7 mg of CB3-I, (the yield for CB3-II was the same as for CB3-I) representing an overall yield of 91% (CB1 and CB2 were recovered in yields of 43.0 and 18.5 mg, respectively, from the initial fractionation, before each was rechromatographed further). Calculation of the molar yields of each fragment showed that for every 1 μmole of CNBr-treated OT after fractionation 2 μmoles of each fragment are recovered. These results clearly indicate that each fragment is liberated in a duplicate fashion.

N-Terminal Analysis of the CNBr Fragments. Cyanate determination of the N-terminal amino acids for each fragment gave the following results: CB1, alanine; CB2, glycine. CB3-I gave inconclusive results because of insolubility of this fragment in the *N*-ethylmorpholine–acetate buffer (pH 8.5), even in the presence of urea. The CB3-II preparation, however, did not present this problem and was, therefore, used in place of CB3-I. Two N-terminal amino acids (presumably one

TABLE II: Molecular Weight of CNBr Fragments of Ovotransferrin.

Fragment	Molecular Weight by		
	Composition Analysis	Gel Filtration	Osmotic Pressure[a]
CB1	21,000	20,000	21,200
CB2	9,400	10,400	
CB3-I	7,000	7,500	

[a] Molecular weight was determined from a graph of osmotic pressure *vs.* concentration extrapolated to zero concentration.

TABLE III: Relative Recoveries of CNBr Fragments from Ovotransferrin.

Fragment	Arbitrary Area Units[a]	Molecular Weight (by Composition Analysis)	Area/ Molecular Weight
CB1	176,000	21,000	8.4
CB2	81,000	9,400	8.6
CB3-I	58,000	7,000	8.3

[a] Determined by planimetry.

per component of CB3-II), leucine and phenylalanine, were detected. It was, therefore, concluded that the N terminal of CB3-I must be either leucine or phenylalanine. Dansylatin of fragments and thin-layer chromatography determination of the dansylamino acids, as well as Edman degradation followed by gas chromatography identification of phenylthiohydantoin amino acids, substantiated this conclusion.

Reduction and Carboxymethylation of CNBr Fragments. Reduction and carboxymethylation of each of the fragments was judged complete by noting the quantitative conversion of half-cystine residues into *S*-carboxymethylcysteine. The reduced-carboxymethylated fragments had the same composition as the intact fragments, and upon electrophoresis on acrylamide gel slabs (under the same conditions described earlier), each still showed only a single band with a mobility less than its native counterpart.

Search for Small Peptides. Although the total recovery of material represented by the three CNBr fragments was 95% of the starting material (after the initial fractionation), the possibility that a small peptide (or peptides) was not detected could not be excluded. To test this possibility CNBr-treated OT was fractionated on a 2 × 84 cm bed of Bio-Gel P-4 (200–400 mesh), using 0.2 M acetic acid as the eluent. Fractions were collected for a total of 1.5 column volumes (400 ml). Small groups of fractions were pooled and lyophilized and then hydrolyzed in 6 N HCl prior to amino acid analysis. Small amounts of aspartic acid, threonine, serine, glutamic acid, glycine, and alanine were detected, but not in amounts sufficient to suggest the presence of a newly discovered small peptide.

Discussion

Cleavage of OT with cyanogen bromide resulted in the recovery of three unique polypeptide fragments in a stoichiometric ratio of 1:1:1. The sum of the molecular weights and the total amino acid and carbohydrate contents of the fragments were one-half that of the native protein. The quantitative recovery of the three fragments accounted for 91% of the starting material, and quantitation of the recovery of each fragment revealed that 2 moles of each fragment was recovered for every 1 mole of OT cleaved and fractionated. Since OT is a single polypeptide chain (Bezkorovainy and Grohlich, 1967; Greene and Feeney, 1968; Mann *et al.*, 1970), these data clearly indicate that OT is composed of duplicate (possibly identical) segments joined to form a single polypeptide chain.

The extent of conversion of methionine into homoserine and homoserine lactone varied from 95 to 99% as determined by amino acid analysis. The variation in the extent of conversion was traced to the nature of the CB3 fragment in different preparations. In most preparations conversion and cleavage were complete, producing fragments denoted as CB1, CB2, and CB3-II (see Figure 2). In a limited number of preparations, however, a single polypeptide fragment, CB3-I, was found in place of CB3-II. The molecular weight of CB3-I was estimated to be approximately twice that of CB3-II. The amino acid compositions of CB3-I and CB3-II were identical, except for methionine and homoserine lactone–homoserine content. While CB3-II contained two residues of homoserine lactone, CB3-I contained one residue of homoserine lactone and one residue of unmodified methionine. The causes of noncleavage at the methionine residues have been reported previously in the case of other proteins (Adelstein and Kuehl, 1970; Schroeder, 1969; Narita and Titani, 1968), and include such factors as conversion of methionine into the sulfoxide, and the proximity of serine or threonine to the methionine residues. The former results in a CNBr-resistant derivative of methionine while the latter results in the conversion of methionine into homoserine without subsequent cleavage. Because of the identical amino acid compositions of CB3-I and CB3-II (except for methionine and homoserine content), and because the only difference in the fractionation of different batches of CNBr-treated OT was found in the nature of the CB3 fragment (CB1 and CB2 were always constant with respect to elution volume and quantitative yield), it is proposed that CB3-II represents the complete cleavage products of CB3-I.

On the basis of 8 methionine residues for OT, CNBr cleavage should have theoretically produced 9 fragments. The total homoserine–homoserine lactone content of the CNBr fragments accounted for the conversion of 8 methionine residues. We can also quantitatively account for the actual and potential production of 8 moles of fragments for every mole of OT cleaved. Therefore, the possibility exists that a small oligopeptide was produced, but was undetected by our separation procedure. This is especially significant in view of our experimental data which indicate the presence of 1 mole of homoserine lactone per each fragment. Assuming a duplicate structure for OT the CNBr fragment from the C-terminal region should be theoretically devoid of homoserine lactone. One explanation for this discrepancy would be to assume a methionine residue situated close to the C-terminal position. The cleavage of this residue would produce a small oligopeptide and a new C-terminal homoserine lactone.

The present data are insufficient for assigning a C-terminal position for any of the fragments. One of the CB1 fragments, however may be temporarily assigned the N-terminal position since alanine is known to be the N-terminal amino acid of OT (Fraenkel-Conrat and Porter, 1952).

Detection of only one kind of N-terminal residue, and production of a homogeneous reduced-carboxymethylated derivative, for each fragment strongly indicates that the methionine residues must be located outside of the S–S-containing polypeptide chains comprising each fragment.

The question of absolute sequential homology of the duplicate subunits is a matter of speculation at present. The recent data of Elleman and Williams (1970) showed the isolation of 34 sequentially unique cysteic acid containing peptides from OT, which would indicate that OT does not contain a repeating sequence. However, our finding of apparently identical duplicate fragments is based predominantly on the molecular size, shape, and charge distribution, which indicate a homogeneous population of molecules for each fragment. There

still remains a possibility of sequential differences in a limited number of amino acids (possibly neutral) between the duplicate fragments. The knowledge of the primary structure of each fragment will resolve this question.

It is also found that the distribution of carbohydrate on the CNBr fragments is inconsistent with the previous work of Williams (1968), who found that in OT the bulk (85%) of carbohydrate residues (presumably as a single oligosaccharide chain) were attached to a unique peptide containing one residue of aspartic acid. The finding of mannose and glucosamine in both CB1 and CB2 clearly indicates that these residues are distributed on different loci of the OT molecule. However, the manner of distribution of these residues in the native protein is a matter of speculation. Several possibilities exist. (1) All the carbohydrate is located on one-half of the protein molecule at two different loci, corresponding to CB1 and CB2. (2) The carbohydrate is distributed equally between both halves of the molecule at four loci, corresponding to CB1 and CB2 and their duplicated portions CB1′ and CB2′. (3) The carbohydrate is found at one location CB1 (or CB1′), on one-half of the molecule and at a second location, CB2 (or CB2′), on the other half of the molecule. The present data do not allow us to distinguish between these alternatives.

It remains now to demonstrate how the ovotransferrin molecule is biosynthesized. Genetic economy would dictate that both halves of the protein are synthesized individually from the same segment of DNA, with the two resulting polypeptide fragments (mol wt 38,000) undergoing subsequent fusion to form a single polypeptide chain (mol wt 76,600). It may be, however, that partial gene duplication of allelic genes has occurred with the result that a single polypeptide chain of molecular weight 76,600 is synthesized from a single segment of DNA. Such duplications have been reported previously, in the case of γ-globulin light chains (Lennox and Cohn, 1967) as well as other nonheme iron proteins, such as the ferredoxins and rubredoxins (Tsunoda *et al.*, 1968; Weinstein, 1969). The latter proteins have been postulated to have evolved from a common precursor of roughly half their present size by gene duplication. A similar genetic mechanism may be responsible for the evolution of ovotransferrin from an evolutionary antecedent of molecular weight 38,000 containing one iron-binding site.

Acknowledgments

The authors wish to thank Dr. D. Baum, University of Colorado Medical School, Boulder, Colo., for his assistance in conducting the membrane osmometry experiments. Appreciation is also due Dr. K. T. Yasunobu, of University of Hawaii, Honolulu, Hi., Department of Biochemistry and Biophysics, in whose laboratory the identification of phenylthiohydantoin amino acids by gas chromatography procedure was conducted.

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