THE COMPLETE AMINO ACID SEQUENCE OF THE FOLATE-BINDING PROTEIN FROM COW'S MILK

by

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The complete amino acid sequence of the folate binding protein from cow's milk has been determined by automated sequencing of fragments obtained by cleavage with cyanogen bromide, trypsin, *S. aureus* V8 protease and clostripain. The molecule consists of a single polypeptide chain of 222 amino acid residues and eight disulphide bridges. The molecular weight of the protein part is 25,700. The folate binder contains carbohydrate which gives it a final molecular weight of about 30,000. No sequence homology with other proteins has been observed.

1. INTRODUCTION

Proteins which specifically bind folate have been isolated from various body fluids and tissues of mammals (1, 7, 12, 14, 15, 16, 18, 19, 20) and also from bacteria (9). In order to understand the function of these proteins it is important to know their structures. In a previous communication we have shown the partial amino acid sequence of the folate binding protein from cow's milk based on the cyanogen

bromide fragments (16). Homology between this protein and the high and low molecular weight folate binding protein from human milk has been demonstrated (17). The present communication shows the complete amino acid sequence of the folate binding protein from cow's milk based on sequence determination of fragments generated by digestion with trypsin, *Streptomyces aureus* V8 protease, and clostripain.

Abbreviations: CNBr = Cyanogen bromide; CPD-Y = Carboxypeptidase Y; DPC = Diphenylcarbamy; DTT = Dithiothreitol; FBP = Folate binding protein; HEPES = N-2-Hydroxyethylpiperazine-N'-2-ethane-sulfonic acid; HPLC = High performance liquid chromatography; 2-PE = 2-pyridylethylated; PTH = Phenylthiohydantoin; TFA = Trifluoroacetic acid.

2. MATERIALS AND METHODS

2. 1. Materials

Folate binding protein FBP was prepared from cow's milk as described in ref. (16). DPC-trypsin and clostripain was from Sigma, St. Louis, U. S. A. *S. aureus* V8 protease from Miles Laboratories, U. S. A. Carboxypeptidase Y was prepared at the Carlsberg Laboratory. Biogel P6, P10, P30 and P60 were purchased from Biorad Laboratories, Richmond, U. S. A. All reagents for the sequencer were from Rathburn, Walkerburn, U. K. except ethyl acetate which was obtained from Merck, Darmstadt, F. R. G. and Polybrene which was from Pierce Rochford, U. S. A. All other reagents used were analytical grade products.

2. 2. Methods

2. 2. 1. Cyanogenbromide fragments

The preparation and purification of cyanogen bromide fragments from reduced and vinyl-ethylated FBP have already been described (16). The four resulting fragments are numbered in the order in which they appear in the sequence (CNBr-I to CNBr-IV).

2. 2. 2. Analytical methods

Amino acid analysis, automated amino acid sequence determination and carbohydrate determination were conducted as described in ref. (10).

2. 2. 3. Digestion with carboxypeptidase Y

To 40 moles of CNBr-IV in one ml 0.2 M-ammoniumacetate pH 5.0 was added CPD-Y in a molar ratio of 1:200. At suitable time-intervals aliquots were removed, the reaction stopped by addition of 1M-HCl and the sample then applied to the amino acid analyzer.

2. 2. 4. Digestion with DPC-Trypsin

FBP, ca 12 mg, was dispersed in 1 ml of 0.5 M-ammonium bicarbonate and acetylated by addition of 3×25µl acetic anhydride at intervals of 10 minutes. After the last addition the reaction mixture was left for two hours at room

temperature in order to hydrolyze excess of reagent. Then, 50 µl of DPC-trypsin (1 mg×ml⁻¹) was added and the reaction mixture left for two hours with occasional adjustment of the pH to between 8 and 9. Following centrifugation the supernatant was applied to a column of Biogel P30 (0.9×100 cm) equilibrated with 30% acetic acid and eluted with the same solvent. The eluate was monitored at 280 nm and the relevant peaks pooled and lyophilized. The individual peaks were then reduced with mercaptoethanol and treated with 2-vinylpyridine (8) and finally fractionated under the same conditions as above.

In a different experiment the tryptic cleavage was conducted at pH 8.0 in a 0.1 M-phosphate buffer and treated as above.

2. 2. 5. Digestion with clostripain

CNBr-III (8 mg) of reduced, vinylethylated FBP was digested with clostripain at pH 5.5 in 0.05 M-HEPES buffer (0.05 M in CaCl₂, 2.5×10⁻³ M in DTT and 4 M in urea) overnight at 37 °C. After centrifugation the supernatant was gel filtered on a Biogel P6 column (1.6×85 cm) in 30% acetic acid and the elution profile monitored at 280 nm.

2. 2. 6. Digestion with *S. aureus* V8 protease

FBP (46 mg) was dissolved in 10 ml acetate buffer pH 4.0 and 0.5 mg *S. aureus* V8 protease added. The reaction mixture was left overnight at room temperature and then lyophilized. Separation of peptides was initially made by gel filtration on Biogel P30 (2.5×90 cm) in 30% acetic acid and appropriate peaks pooled and lyophilized.

Further purification was obtained by reduction and vinylethylation as described in 2. 2. 4 followed by gel filtration in the system just described.

3. RESULTS

Treatment of unreduced FBP with DPC-trypsin was made in the hope that the resulting peptides would separate upon gel filtration in such a way that some information would be obtained about the distribution of disulphide

bridges. This was unfortunately not the case since the elution pattern from gel filtration on Biogel P30 showed one major peak of high molecular weight and low molecular weight peptides of no interest for the completion of the sequence or the study of disulphide bridges. Consequently, the main peak was reduced and vinylated. Gel filtration on Biogel P30 resulted in five peaks of which the last one was attributed to reagents (see Figure 1). Peak T-2 was rechromatographed twice on Biogel P60 resulting in a peptide the sequence of which began: Val₁₀₉-Leu₁₁₀-Gly₁₁₁- and which was followed 31 steps through Gly₁₃₉. The numbering of the amino acids is that of the complete sequence to be discussed later (see Figure 5).

Figure 1. Gelfiltration of tryptic peptides of FBP after reduction and vinylilation on Biogel P30 (0.9×100 cm) in 30% acetic acid. Flow-rate: 4.5 ml×hr⁻¹. Fractions: 1 ml.

Table I.

Amino acid composition of folate binding protein from cow's milk and its cyanogen bromide fragments.

Amino acid	FBP			CNBr-I	CNBr-II	CNBr-III		CNBr-IV	
Asn	12 ¹⁾	2 ²⁾	3 ³⁾	1 ¹⁾	2 ¹⁾	6 ¹⁾		3 ¹⁾	
		22.6 (23)	25		7.0 (7)	10.0 (10)		3.9 (4)	
Asp	10			1	4	4		1	
Thr	10	10.2 (10)	13	2	1 1.2 (1)	5 4.7 (5)		2 2.1 (2)	
Ser	17	17.1 (17)	16		5 5.0 (5)	10 8.7 (9)		2 2.1 (2)	
Gln	9			1	1	5		2	
		23.6 (24)	24		5.4 (5)	12.0 (12)		5.4 (5)	
Glu	14				4	7		3	
Pro	16	16.0 (16)	16	2	3 3.4 (3)	7 7.1 (7)		4 4.1 (4)	
Gly	11	11.4 (11)	13		2 2.1 (2)	6 6.0 (6)		3 3.0 (3)	
Ala	14	13.3 (13)	15	3	4 4.1 (4)	5 4.9 (5)		2 2.1 (2)	
Cys	16	11.0 (11)	15	1	4 3.8 (4)	11 11.8 (12)			
Val	8	7.9 (8)	10	1	1 1.1 (1)	5 4.9 (5)		1 1.0 (1)	
Met	3	2.9 (3)	3	1	1 n.d. ⁴⁾	1 n.d. ⁴⁾			
Ile	7	6.5 (7)	8		2 2.0 (2)	4 3.1 (3)		1 0.9 (1)	
Leu	9	9.6 (10)	12	2	2 2.4 (2)	5 4.5 (5)			
Tyr	9	8.9 (9)	9		2 2.0 (2)	6 5.7 (6)		1 1.0 (1)	
Phe	8	7.6 (8)	8		1 1.6 (2)	4 4.7 (5)		3 2.7 (3)	
His	8	8.0 (8)	9		4 4.5 (5)	4 3.5 (5)			
Lys	12	12.5 (13)	13		5 5.5 (6)	7 8.0 (8)			
Arg	15	14.4 (14)	15	3	2 2.3 (2)	9 7.3 (7)		1 1.0 (1)	
Trp	11	8.5 (9)	4		2 n.d.	8 n.d.		1 n.d.	
Glucosamine		5.9 (6)	4		+++	+++			
Total	219+3 ⁵⁾	214	232	18	52+2	119+1		30	

1) From the sequence. 2) From amino acid analysis. 3) From ref. (14). Recalculated to fit M.W. 25.700.

4) Homoserine and homoserine lactone present. +++ indicates positive carbohydrate reaction (6).

5) The numbers +3, 2 and 1 in the total sum of amino acids refer to the vacant positions in the sequence.

Figure 2. Gel filtration of CNBr-III digested with *S. aureus* V8 protease on Biogel P30 (0.9×165 cm) in 0.1 M-pyridine-acetate buffer, pH 4. Fractions: 1 ml.

Peak T-4 (Figure 1) contained Met and sequence determination showed two peptides one of which began Gly₁₈₅-Ser₁₈₆-Gly₁₈₇ while the other started Cys₁₈₉-Ile₁₉₀-Gln₁₉₁ showing incomplete cleavage of the Arg₁₈₈-Cys₁₈₉ bond. Both sequences were followed through Glu₂₀₄.

From a tryptic cleavage of FBP conducted in phosphate buffer followed by opening of the disulphide bonds and gel filtration a methionine containing peak was isolated which also consisted of two peptides. One of these, Lys₄₂-Asn₄₃-Ala₄₄, was known and the other could therefore be followed 12 step starting Phe₆₄-Asn₆₅-Trp₆₆.

The purified cyanogen bromide fragments (except the small N-terminal CNBr-I the sequence of which was already known) were used as starting material for the generation of peptides necessary to complete the sequence of FBP. In Table I is shown the amino acid compositions of CNBr-II, CNBr-III and CNBr-IV as obtained from the sequence and from amino acid analyses. In all three cases a fair agreement between the two sets of data is obtained.

Digestion of CNBr-III with clostripain and gel filtration gave rise to four peaks (not shown). One was sequenced 20 steps and shown to contain two peptides. These were identified as Thr₁₂₈-Ser₁₂₉-Tyr₁₃₀ and Phe₁₅₈-Asp₁₅₉-Phe₁₆₀.

The first one was known while the last one in part was new.

CNBr-III was cleaved with *S. aureus* V8 protease and gel filtration on Biogel P30 resulted in six peaks as shown in Figure 2. Rechromatography of peak S III gave a clean peptide which was sequenced 39 steps starting at Asp₁₂₅-Cys₁₂₆-Arg₁₂₇. Peak S VI was sequenced without further purification 17 steps beginning at Ile₁₇₂-Trp₁₇₃-Ser₁₇₄. Amino acid analyses showed the presence of homoserine and homoserine lactone identifying the peptide as the C-terminal part of CNBr-III.

The remaining peaks S I, S II, S III and S V were all mixtures of peptides without importance for the completion of the sequence. The following expected sequences were identified: Glu₇₃-Pro₇₄-Ala₇₅, Cys₈₉-Ser₉₀-Pro₉₁, Val₁₀₀-Asn₁₀₁-Gln₁₀₂, Asp₁₁₈-Cys₁₁₉-Gln₁₂₀.

Cleavage of CNBr-II with *S. aureus* V8 protease resulted in a series of peptides which were separated as shown in Figure 3. A peptide contained in peak S4 started Ile₅₈-Ser₅₉-Tyr₆₀ and was followed through Asp₆₇. It contained homoserine and homoserine lactone and thus represented the C-terminal part of CNBr-II. It had the following amino acid composition: Asp₂, Ser₁, HSer₁, Gly₁, Ile₁, Leu₁, Tyr₂, Phe₁,

Figure 3. Gel filtration of CNBr-II digested with *S. aureus* V8 protease on Biogel P30 (0.9×165 cm) in 0.1 M-pyridine-acetate buffer pH 4. Flow-rate 8.5 ml×hr⁻¹. Fractions: 1.3 ml.

His₁, 2-PECys₁, Lys₁ and Arg₁. The preceding peak S 3 in Figure 3 contained two peptides: Ala₅₄-? -Lys₅₆- and Asp₁₉-Ala₂₀-Lys₂₁- (N-terminal part of CNBr-II). Peaks S1 and S2 were not investigated further.

Cleavage of FBP, with intact disulfide bridges, by *S. aureus* V8 protease upon gel filtration resulted in one peak containing high molecular weight material and one with the amino acid composition: Asp(1), Thr(2), Ser(2), Glu(2), Pro(2), Gly(2) and Ile(1) which is compatible with the C-terminal 12 residues of FBP. Sequencing was possible for nine steps starting at Glu₂₁₁-Asn₂₁₂-Pro₂₁₃-. In this case *S. aureus* protease acted in an unusual way since cleavage has taken place on the N-terminal side of a Glu.

Digestion of CNBr-IV with carboxypeptidase Y released four amino acids in the order shown in Figure 4. The C-terminal sequence of FBP is thus -Pro-Gln-Gly-Ile·COOH.

Construction of the complete sequence (cf. Figure 5). In a previous publication (16) the N-terminal 62 residues were shown based on sequencing of reduced, vinylated FBP and one of the CNBr fragments, CNBr-II. This sequence has been confirmed and positions 52 and 58 identified as isoleucines. Position 53 was erroneously published as Gly while it in fact is

Glu. From CNBr-II digested with *S. aureus* V8 protease a peptide was isolated which began at Ile₅₈ and was sequenced through Asp₆₇. Another peptide found as the main component in a mixture began at Ala₅₄ and was sequenced through Asp₆₇ confirming the sequence. Positions 49 and 55 were vacant in all cases.

The overlap between CNBr-II and CNBr-III was provided by a tryptic peptide isolated from a digest of FBP. It was sequenced 12 steps starting at Phe₆₄ together with an already known sequence starting at Lys₄₂. CNBr-III starting at Glu₇₃ was previously sequenced 30 steps through Gln₁₀₂ (16). This sequence was repeated and extended to include Cys₁₁₉. Part of this sequence was confirmed (Cys₈₉-Leu₁₁₄) by sequencing a peptide obtained by cleavage of CNBr-III with *S. aureus* V8 protease.

The sequence of FBP was extended further by a tryptic peptide obtained by cleavage of FBP which began at Val₁₀₉ and was followed through Gly₁₃₉. The last 15 residues of the sequence was confirmed and extended through Pro₁₆₃ by a peptide obtained from cleavage with *S. aureus* V8 protease. A six residues overlap was obtained by a clostripain derived peptide and the sequence extended through Tyr₁₇₇. A prolongation to

Figure 4. Digestion of CNBr-IV with carboxypeptidase Y in 0.2 M-ammoniumacetate, pH 5.0. Enzyme-substrate ratio: 1:200.

Arg₁₈₈ was made by another *S. aureus* V8 protease derived peptide which started at Ile₁₇₂.

The overlap between CNBr-III and CNBr-IV was obtained by two peptides both isolated from FBP after cleavage with clostripain. One sequence started at Gly₁₈₅ and the other at Cys₁₈₉. Both were sequenced through Glu₂₀₄.

The C-terminal CNBr-fragment has already been published (16) except for the final positions of the Pro and Gln in positions 219 and 220. A peptide purified from the digest of FBP by *S. aureus* V8 protease was sequenced nine steps and included Pro₂₁₉. Digestion of the same peptide with CPD-Y liberated four amino acids including Pro₂₁₉. The Glu originally published based on amino acid composition showed to be a Gln in the sequence.

4. DISCUSSION

Completion of the amino acid sequence of the folate binding protein from cow's milk showed this to be a single peptide chain consisting of 222 amino acid residues with carbohydrate attached at two or three side-chains. As discussed in the publication of the partial sequence the N-terminal sequence was "ragged". Besides the sequence starting at Ala₁, another was observed beginning at Ala₉. In some preparations used in the sequence work cleavage between Arg₁₀ and Thr₁₁ was seen (but never between Arg₅ and Thr₆). These findings point towards a trypsin-like activity in cow's milk (11).

The FBP is a glycoprotein as shown by the phenol-sulphuric acid method (6) and by the presence of glucosamine in amino acid hydrolysates. We found that carbohydrate was present in CNBr-II as well as CNBr-III. CNBr-III has only one unassigned position (no. 141 in Figure 5) and the carbohydrate must therefore be located at this position. Position 143 is occupied by a Thr which suggests that the carbohydrate moiety is attached to an asparagine side-chain in position 141.

In CNBr-II two positions, 49 and 55, are vacant. Of these, position 49 is most likely occupied by a carbohydrate-linked asparagine since a Ser is located in position 51. On the other hand, position 55 must have Ser or Thr if carbohydrate is attached. However, when the

peptides obtained by cleavage of CNBr-II with *S. aureus* V8 protease were separated by gel filtration (see Figure 3) peak S3 which contained a peptide starting at Ala₅₄ was negative with respect to carbohydrate while peaks S1 and S2 were strongly positive. It is possible therefore that position 55 is occupied by an amino acid which is modified in such a way that its PTH-derivative was not identified.

Positions 155 and 156 in the sequence have not been definitely assigned. A peptide obtained by digestion with pepsin, Asn₁₄₇-Phe₁₆₀, was treated with CPD-Y and the release of amino acids agrees with the sequence shown in Figure 5 but the similar yields of His and 2-PECys leave some doubt on their relative position.

Several attempts were made to determine the disulphide bond arrangement in FBP, but without success. Further studies with different approaches are needed to locate the eight disulphide bridges.

A comparison of the amino acid composition based on the sequence with that obtained from amino acid analysis is made in Table I. A reasonable good agreement is seen, the largest deviations found for Cys and Trp. The lack of two Asx residues in the composition derived from the sequence may be due to the two carbohydrate-linked Asn's in positions 49 and 141. The amino acid composition based on amino acid analysis used here deviates slightly from that originally published. A redetermination was made on FBP which had not been previously spray-dried.

The molecular weight of FBP calculated from the sequence is about 25.700 for the protein part. The uncertainty is introduced by the unassigned position 55 in the sequence.

The amino acid composition of FBP from cow's milk determined by SALTER et al. (14) is included in Table I. The values have been recalculated to a molecular weight of 25.700 for direct comparison. Also in this case a fair agreement with the sequence is seen.

Determination of the molecular weight by sedimentation equilibrium centrifugation gave 30.000 +/- 2000 (16). Within these limits the carbohydrate content varies then between 8 and 20%. The determination of carbohydrates in different batches of FBP has given values rang-

ing from 3 to 10%. SALTER et al. (14) have determined the content of the individual carbohydrates in FBP and showed that approximately 40% is contributed by N-acetylglucosamine and N-acetylgalactosamine. These authors found a carbohydrate content of 10%. The phenol-sulphuric acid method used in our studies does not detect the presence of aminosugars and sialic acid. It is conceivable then that part of the deviation in molecular weight determination based on our centrifuge studies and sequence studies can be explained by the content of galactosamine and glucoamine.

SALTER et al. (14) determined the molecular weight to 35.000 from sedimentation equilibrium studies. We are unable to explain the large discrepancy between our and their values but a difference in experimental technique was that they used 6 M-guanidinium chloride as solvent.

Inspection of the amino acid sequence with respect to charge shows an interesting distribution of positive charges. These tend to cluster, the most striking example being Lys₂₁-His₂₂-His₂₃-Lys₂₄, but also Arg₄₁-Lys₄₂, Lys₇₇-Arg₇₈-His₇₉, Arg₁₀₃-Trp₁₀₄-Arg₁₀₅-Lys₁₀₆-Glu₁₀₇-Arg₁₀₈ and His₁₃₇-Lys₁₃₈ illustrate this. It will be interesting to learn if any of these charge-clusters are important for the strong binding of folate or its polyglutamyl derivative.

Prediction of the secondary structure by the CHOU and FASMAN method (5) shows a high (approx. 40%) content of α -helix while the β -structures only amounts to approx. 15%.

Comparison of the amino acid sequence of FBP with those of other milk proteins such as α -lactalbumin (4) and β -lactoglobulin (3) has not revealed any sequence homologies. Comparison with the sequence of tetrahydrofolate reductase from *E. coli* (1) and *L. casei* (1) also failed in showing homology. To our knowledge the amino acid sequence shown here represents a novel family of proteins the only other member hitherto sequenced in part is the one isolated from human milk (17).

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