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Preliminary results for the primary structure of bacterioferritin of *Escherichia coli*

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Bacterioferritins are type-*b* cytochromes which resemble ferritin. Amino acid analysis combined with chemical modification and partial sequence analysis characterize bacterioferritin of *Escherichia coli* in terms of its primary structure. It is a protein composed of one kind of polypeptide chain that commences with methionine and terminates with glutamic acid. The length of the polypeptide chain is, tentatively, 146 residues. Besides the *N*-terminal methionine residue there are three more methionine residues, which yield four CNBr peptides, which have been aligned. The identity of the following positions in the sequence has been ascertained: residues 1–25, 30–37, 83–88, 127–132 and 143–146. No homology with ferritin was found.

INTRODUCTION

The time course of iron uptake in a growing culture of *Escherichia coli* suggests that iron is accumulated and stored before it is utilized in growth (McIntosh & Earhart, 1977). The presence of magnetically ordered aggregates of iron in *E. coli* has been demonstrated, and the molecule harbouring these aggregates has been isolated (Bauminger *et al.*, 1979, 1980). The similarity between the isolated molecule and ferritin was noted in electron-microscopic projections of the molecule and in the symmetry of the molecule as deduced from X-ray-diffraction analysis of single crystals (Yariv *et al.*, 1981). However, bacterioferritin is a cytochrome, and it has been isolated previously from *E. coli* under the name 'cytochrome b_1' (Yariv, 1983). Because bacterioferritin is isolated from the soluble fraction of *E. coli*, we refrain from calling it 'cytochrome b_1' until the primary structure of membranal cytochrome b_1 is known. Bacterioferritins are found in other bacterial genera besides *Escherichia* (see, e.g., Stiefel & Watt, 1979). They are all type-*b* cytochromes and all store magnetically ordered aggregates of iron. They also cross-react with anti-(*E.coli* bacterioferritin) antibody but not with anti-(horse spleen ferritin) antibody (J. Yariv, unpublished work).

The bacterioferritin that we used for sequence analysis was isolated from supernatants of *E. coli* cells disrupted by sonication. It was isolated by precipitation with antibody and by separation of the protein from antibody in an 8 M-urea solution containing 2% (v/v) acetic acid (Yariv *et al.*, 1981). Under such conditions the protein dissociates and emerges from a CL-Sepharose 4B column after the antibody. Extensive dialysis of the isolated red fraction, first in a buffer solution and then in water, reconstitutes the molecule to the ferritin-like structure. Despite relatively small losses in this purification procedure, the yields of the protein are low and correspond, on the average, to 5 mg of protein from 1 kg of wet bacterial paste. Accordingly, analyses were performed on nanomolar quantities of the protein.

EXPERIMENTAL

E. coli grown under anaerobic conditions with nitrate as the electron donor was the source of bacterioferritin used in the present work. Whole serum of an immunized goat was used as antibody. Haem was removed from protein by the procedure of Teale (1959).

Amino acid analyis

The protein (5 μg) or peptide (0.05–0.5 nmol) was hydrolysed with 100 μl of a 2:1 (v/v) mixture of HCl and trifluoroacetic acid containing 0.05% phenol at 166 °C for 25 or 50 min in evacuated and sealed tubes (Tsugita & Scheffler, 1982). The cysteine content was determined after performic acid oxidation of the protein (Hirs, 1956). Tryptophan was analysed by a micro-scale method in which 3 M-mercaptoethanesulphonic acid was used to hydrolyse the protein (Maeda *et al.*, 1984). The amino acid analysis was carried out with a Durum D500 analyser calibrated for 2.5 nmol full-scale deflection on the recorder.

Sequence analysis

C-Terminal sequences were analysed by digestion with carboxypeptidases A, B (Sigma) and P (Takara, Shuzo, Japan) in 0.1 M-pyridine/acetate or pyridine/formate buffers at pH 8.2, 8.2 and 2.5 respectively as described by Schurmann *et al.* (1981). *N*-Terminal sequences were determined by automatic Edman degradation with a Beckman sequenator (890-C). We used the normal 0.1 M-Quadrol program from Beckman with double coupling in the first step. The thiazoline amino acids were converted into amino acid phenylthiohydantoin derivatives by incubating them for 10 min at 50 °C with acetyl chloride/methanol (1:7, v/v). All reagents for the sequenator were obtained from Beckman, except ethyl acetate and butyl chloride, which were from Pierce, and dithioerythritol, which was from Sigma. Polybrene (3 mg) from Pierce was used as a carrier (Wittmann-

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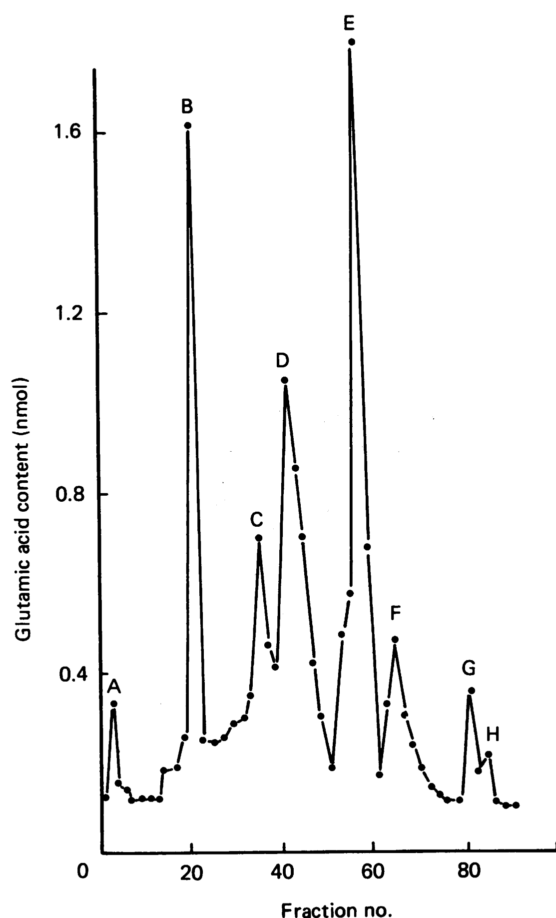

Liebold, 1973). The phenylhydantoin amino acids were analysed by h.p.l.c. (Hunkapiller & Hood, 1983).

S-Carboxymethylation

S-Carboxymethylation of the protein (300 nmol) was performed as described by Hirs (1967). Iodo[^{14}C]acetic acid (5 μCi/μmol) was purchased from New England Nuclear Corp. and iodoacetic acid was from Serva Feinbiochemica. The modified protein was recovered by gel filtration on a Bio-Gel P-10 column (Bio-Rad laboratories; 0.8 cm × 80 cm, 100–200 mesh) equilibrated with 70% (v/v) formic acid.

CNBr cleavage of modified protein

Carboxymethylated protein (200 nmol) was dissolved in 500 μl of 70% formic acid and CNBr was added in an approx. 50-fold excess over the methionine concentration. The reaction was allowed to proceed for 72 h at

Fig. 1. Fractionation of CNBr-cleaved bacterioferritin

Bacterioferritin (200 nmol), carboxymethylated and cleaved by CNBr, was fractionated on a column of SP-Sephadex C-25. A 50 μl portion, taken from every other tube, was hydrolysed and the values for glutamic acid are plotted against tube number. Fractions B–E were collected and further purified by molecular sieving; fraction C was purified on a Bio-Gel P-6 column (0.8 cm × 80 cm), equilibrated with 70% formic acid, fractions E and D on a Bio-Gel P-10 column, and fraction B on a Bio-Gel P-4 column. The amino acid compositions of these fractions are given in Table 1, where I = C, II = E, III = D and IV = B.

Table 1. Amino acid composition of bacterioferritin

	Number of residues in:					
	CNBr peptide				Protein	
	I	II	III	IV	By summation	By direct analysis
Asp	5	7	7	2	21	21
Thr	1	2	1	0	4	4
Ser	1	3	2	0	6	6
Glu	5	7	5	5	22	21
Pro	0	1	0	0	1	1
Gly	2	4	4	4	14	11
Ala	1	3	3	1	8	8
Val	4	3	2	0	9	5
Met	1	1	1	0	4*	4
Ile	3	3	3	1	10	8
Leu	4	6	6	2	18	18
Tyr	1	2	2	1	6	6
Phe	0	1	1	0	2	2
His	1	2	1	0	4	3
Lys	1	2	2	0	5	7
Arg	1	2	2	1	6	7
Cys	2	0	2	0	4	4
Trp	0	2	0	0	2	2
Total...	33	51	44	17	146*	138

* Includes *N*-terminal methionine.

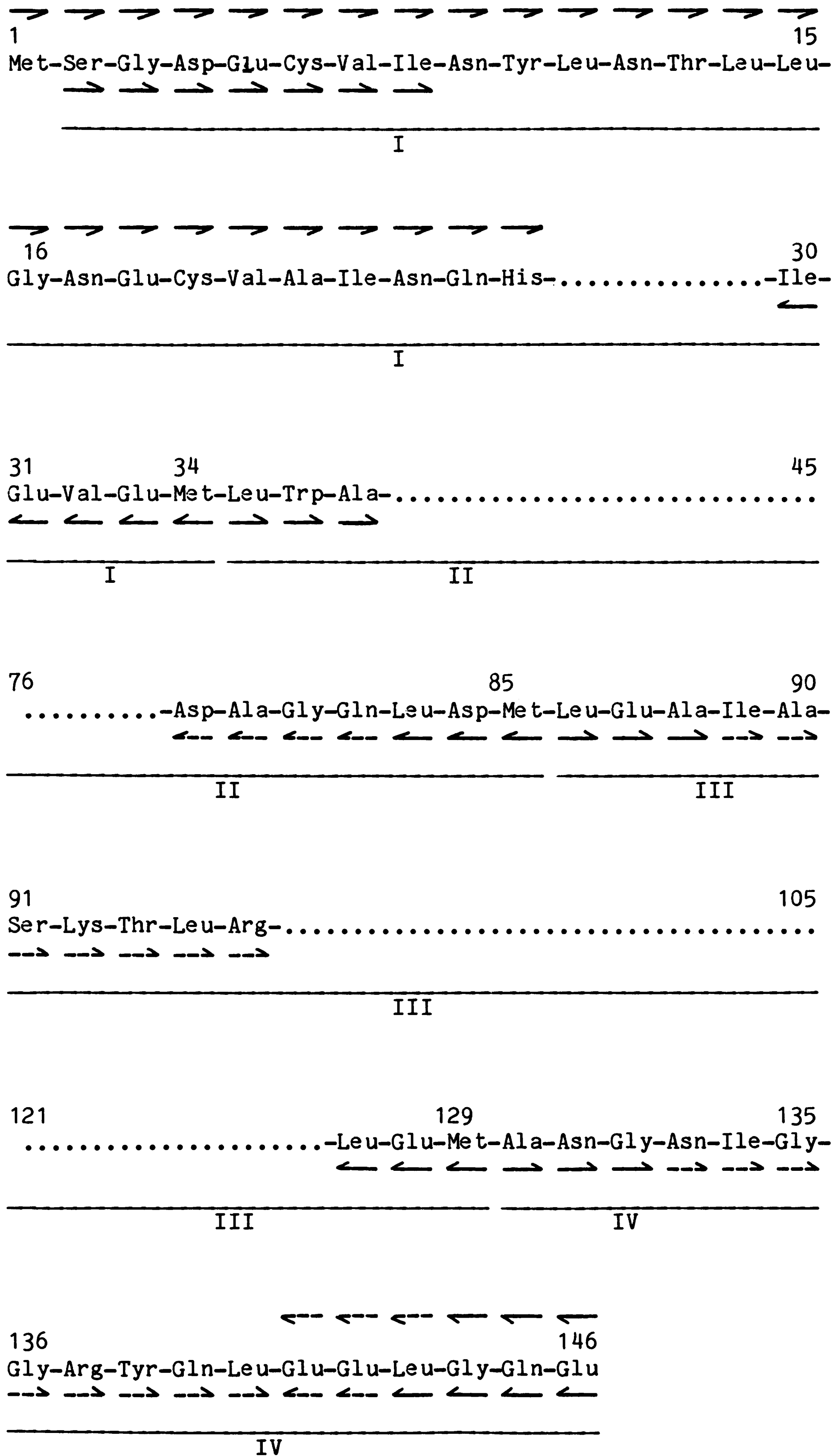

Fig. 2. Partial amino acid sequence of bacterioferritin

Key to symbols: ⟶, Edman degradation of protein; ⟶, Edman degradation of peptides; ⟵, carboxypeptidase A digestion of protein; ⟵, carboxypeptidase digestion of peptides; ----, tentative, unconfirmed, data; I–IV, CNBr peptides.

20 °C, then the sample was evaporated to dryness. The product was fractionated on a column of SP (sulphopropyl)-Sephadex C-25 (Pharmacia; 0.6 cm × 50 cm) with a gradient of linearly increasing pH and ionic strength in pyridine/acetate buffer (Maeda *et al.*, 1982).

RESULTS

The amino acid content of bacterioferritin as obtained by direct analysis is compared with the sum of amino acid contents of the isolated CNBr peptides in Table 1. There is generally an excellent agreement between these values.

Analysis of the products of CNBr cleavage of the protein is presented in Fig. 1. The fractions represented by letters B–E contain more than 80% of total material applied to the column. These fractions correspond to fractions I–IV in the primary-structure map of the protein presented in Fig. 2 (B = IV, C = I, D = III, E = II), which summarizes the sequence information now available. Fractions I–IV were collected and further purified: fraction I on a Bio-Gel P-6 column (0.8 cm × 80 cm) equilibrated with 70% formic acid, fractions II and III on a Bio-Gel P-10 column, and fraction IV on a Bio-Gel P-4 column. The amino acid compositions of these fractions are listed in Table 1.

Alignment of peptides I and IV in the sequence is straightforward from comparison of the sequences at the termini of the protein with those of the isolated peptides. Peptides II and III were aligned with the help of the amino acid composition of an isolated tryptic peptide that corresponds to residues 79–95 in the proposed sequence.

The peptides present in the minor fractions A, F, G and H were not characterized, since the whole sequence was considered to be accounted for by the peptides isolated in good yield from fractions B, C, D and E. However, the possibility that the peptides present in fractions A, F, G and H represent the presence of a second subunit cannot be formally excluded.

DISCUSSION

Our results provide strong evidence that the bacterioferritin molecule is assembled from one kind of polypeptide chain. At present it is not possible to equate this chemical subunit with the crystallographic subunit (J. M. A. Smith, G. F. Ford, P. M. Harrison, J. Yariv & A. J. Gilboa, unpublished work). There are 24 such subunits in the molecule and they are related by 432 symmetry. The volume of 65 nm^3 (65000 $Å^3$) with which the crystallographic subunit is related in the crystal could equally well accommodate one or two polypeptide chains of 16 kDa, the approximate mass of the chemical subunit that we calculate from its composition.

The similarity between the quaternary structure of bacterioferritin and that of ferritin has already been referred to. The polar indexes of these proteins are also similar: 47 for bacterioferritin of *E. coli* and for horse spleen ferritin. However, comparison of the partial amino acid sequence of bacterioferritin, which we publish here, with the amino acid sequence of horse spleen ferritin (Heusterspreute & Crichton, 1981) failed to show any homology between these two molecules. It is possible that, for the purpose of storage of iron in an innocuous and readily available form, similar molecules were evolved separately in the two kinds of organisms, ferritin in the eukaryotes and bacterioferritin in bacteria. If this is the case, it will make the study of bacterioferritin and ferritin structures highly rewarding.

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