Isolation of Pellicular Cobalamin-binding Proteins of the Cobalamin Uptake System of *Euglena gracilis*

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Euglena gracilis pellicle, separated by sucrose density gradient centrifugation, had a high cobalamin (Cbl) binding activity. Of the Cbl binding protein 65% was solubilized from *E. gracilis* pellicle by using 0.1% SDS/2 M-urea and the residual 35% by using 1% (w/v) SDS. Both of the Cbl binding protein fractions showed a broad pH dependence for the binding of Cbl. No evidence for the involvement of SH-groups or metal ions in Cbl binding was obtained. The K_s values for cyanocobalamin of the proteins solubilized with 0.1% SDS/2 M-urea and with 1% SDS were 0.22 and 0.19 nM, respectively. The M_r of a Cbl binding polypeptide of each protein was estimated to be 38 000 by SDS-PAGE. The Cbl binding protein solubilized with 0.1% SDS/2 M-urea was purified to homogeneity. Inhibition experiments on Cbl uptake in *E. gracilis* cells, using an antibody against the Cbl binding protein solubilized with 0.1% SDS/2 M-urea, showed that the Cbl binding proteins solubilized with 0.1% SDS/2 M-urea and 1% SDS take part in the slower secondary phase and in the initial rapid phase of Cbl uptake in *E. gracilis*, respectively.

INTRODUCTION

The mechanism of cobalamin (Cbl) uptake has been studied extensively in mammalian and bacterial cells. In mammals, especially humans, several studies suggest that Cbl bound to transcobalamin-II (TC-II), one of the plasma carrier proteins, is bound to the cell surface receptor and internalized by pinocytosis (Seligman & Allen 1978; Youngdahl-Turner *et al.*, 1979). In *Escherichia coli* Cbl uptake has been reported to be a biphasic process, consisting of Cbl binding to a cell surface receptor, which is a rapid initial phase, (Digirolamo & Bradbeer, 1971) and active transport coupled to metabolic energy, which is a slower secondary phase (Bradbeer & Woodrow, 1976). Some Cbl binding proteins have been purified from the cell envelope and from the periplasmic space of *E. coli* and have been well characterized (Kadner, 1978; Taylor *et al.*, 1972).

The kinetics of Cbl uptake in the protozoa *Euglena gracilis* Z (Sarhan *et al.*, 1980) and *Ochromonas malhamensis* (Bradbeer, 1971) are biphasic, as in *E. coli*. There is no other detailed information available on Cbl uptake in these organisms. *E. gracilis* Z, which requires Cbl for growth, is suitable for the preparation of Cbl-limited cells (Ross, 1952) and is thus useful for the elucidation of the Cbl membrane transport mechanism. In the present work we separated two Cbl binding proteins from the *E. gracilis* pellicle, purified one of them and studied some properties of the proteins. We also investigated their physiological roles in the Cbl system using an immunological approach.

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Abbreviations: Cbl, cobalamin; TC-II, transcobalamin-II; CN-Cbl, cyanocobalamin; Ado-Cbl, 5'-deoxyadenosylcobalamin; Me-Cbl, methylcobalamin; OH-Cbl, hydroxocobalamin; 2-AMP-Cbl, 2-amino-2-methylpropanolylcobalamin; IF, intrinsic factor.

METHODS

Organism and growth. *E. gracilis* SM-ZK, a streptomycin-bleached mutant of *E. gracilis* Z, was cultured for 5 d at 27 °C at 2000 lx in a Cbl-limited (0.05 µg Cbl l⁻¹) Koren–Hutner medium (Koren & Hutner, 1967).

Preparation of pellicle. Pellicle was prepared by the modified method of Hofman & Bouck (1976). All procedures were done at 2–4 °C. *Euglena gracilis* cells (100 g wet wt) were suspended in 500 ml 100 mM-sodium phosphate buffer, pH 7.2, containing 0.3 M-sucrose and disrupted by sonic oscillation. The homogenate was centrifuged at 3000 g for 30 min. The precipitate, which contained the pellicle, was suspended in 50 ml of the same buffer, and the suspension was applied to a stepwise density gradient of 1.9, 1.4 and 1.0 M-sucrose in 100 mM-sodium phosphate buffer, pH 7.2, which was centrifuged at 10000 g for 30 min. The fraction banding between 1.9 and 1.4 M-sucrose, which contained most of the pellicle, was collected and diluted with 100 mM-sodium phosphate buffer, pH 7.2, until the sucrose concentration reached about 0.3 M. The density gradient centrifugation step was then repeated and the resulting preparation used as purified pellicle.

Cbl binding assay. Cbl binding activity was assayed by an equilibrium dialysis method. The dialysis bag containing 100 µl of sample was equilibrated with 10 ml 10 mM-sodium phosphate buffer, pH 7.2, containing 0.5 M, 0.0002% KCN and 10 nM-[³H]cyanocobalamin (CN-Cbl) at 4 °C for 24 h. After equilibrium was reached, the radioactivity of the solutions (50 µl) inside and outside the bag was determined with a liquid scintillation counter (Aloka, LSC 903). The amount of Cbl bound to protein was calculated by subtracting the radioactivity in the solution outside the bag from that in the solution inside the bag. The specific activity is expressed as the amount of Cbl (mg protein)⁻¹.

Partial purification of pellicular Cbl binding proteins. The 0.1% SDS/2 M-urea and 1% SDS fractions, extracted from the purified pellicle, were individually applied to a column (1.5 × 60 cm) of Sephadex G-25 equilibrated with 10 mM-sodium phosphate buffer, pH 7.2, containing 0.5 M-urea, and eluted with the same buffer at 20 °C to remove SDS from the proteins. Each void fraction was applied to a column (1.5 × 10 cm) of DEAE-Bio-Gel A equilibrated with the same buffer. Each column was washed with 50 ml of the buffer and eluted with 300 ml of a linear (0–0.4 M) NaCl gradient in the same buffer. Active fractions were pooled and concentrated using polyethylene glycol (PEG) 20000. The concentrated solutions were applied to a column (1.5 × 70 cm) of Sephacryl S-200 equilibrated with 10 mM-sodium phosphate buffer, pH 7.2, containing 0.5 M-urea and eluted with the same buffer. Each active fraction was combined, concentrated with PEG 20000, and stored at –20 °C until use. All procedures were done at 4 °C unless otherwise specified.

Purification of the Cbl binding protein solubilized with 0.1% SDS/2 M-urea. All purification procedures were done at 2–4 °C unless otherwise specified. The purified pellicle (50 mg protein) was suspended in 1 ml 100 mM-sodium phosphate buffer, pH 7.2, containing 0.1% SDS/2 M-urea. The suspension was incubated with stirring at 30 °C for 1 h and then centrifuged at 12000 g for 10 min. The precipitate was re-extracted under the same conditions and the combined supernatant fractions (2 ml) were applied to a column (1.5 × 60 cm) of Sephadex G-25 equilibrated with 10 mM-sodium phosphate buffer, pH 7.2, containing 0.5 M-urea, and eluted with the same buffer at 20 °C to remove SDS from the solubilized fraction. The void fractions were brought to 50% saturation with (NH₄)₂SO₄ and the precipitate was collected by centrifugation at 12000 g for 30 min and dissolved in 5 ml 10 mM-sodium phosphate buffer, pH 7.2, containing 0.5 M-urea. The solution, after dialysis overnight against 1 litre of the same buffer, was applied to a column (1.5 × 25 cm) of DEAE-Bio-Gel A equilibrated with 10 mM-sodium phosphate buffer, pH 7.2, containing 0.5 M-urea. The column was washed with 100 ml of the same buffer and eluted with 600 ml of a linear (0–0.4 M) NaCl gradient in the same buffer. Active fractions, eluted at 0.1 M-NaCl, were collected and concentrated to a final volume of 2 ml using PEG 20 000. The solution was applied to a column (1.5 × 70 cm) of Sephacryl S-200 equilibrated with 10 mM-sodium phosphate buffer, pH 7.2, containing 0.5 M-urea, and eluted with the same buffer. Active fractions were collected and applied to a column (1.5 × 20 cm) of CM-cellulose equilibrated with the same buffer. The column was washed with 100 ml of the buffer and eluted with 600 ml of a linear (0–0.4 M) NaCl gradient in the same buffer. Active fractions were collected and concentrated to a final volume of 5 ml using PEG 20000. The concentrated solution was stored at –20 °C until use.

Antibody against pellicular Cbl binding protein. Purified pellicular Cbl binding protein (0.1 mg), suspended in 0.2 ml 10 mM-sodium phosphate buffer, pH 7.2, containing 0.9% NaCl, was emulsified with 0.2 ml of Freund's complete adjuvant. A male mouse (body weight about 50 g) was injected intradermally with 0.4 ml of the emulsion at multiple sites. A booster injection of 0.1 mg of pellicular Cbl binding protein in 0.2 ml of Freund's complete adjuvant was given 3 weeks later at the same sites. Two days after the booster injection, the mouse was bled and serum was prepared by the standard method. The IgG fraction was isolated by (NH₄)₂SO₄ precipitation and DEAE-cellulose ion exchange chromatography, and concentrated by (NH₄)₂SO₄ precipitation. The final precipitate was dissolved in 10 mM-sodium phosphate buffer, pH 7.2, containing 0.9% NaCl and dialysed against the same buffer.

Assay for inhibition of Cbl uptake in *E. gracilis* cells by the antibody against pellicular Cbl binding protein. *E. gracilis* cells, grown for 4 d in the Cbl-limited (0.05 µg Cbl l⁻¹) medium, were washed twice with 10 mM-sodium phosphate buffer, pH 7.2, containing 0.9% (w/v) NaCl and shaken at 27 °C for 3 h in 50 ml of the same buffer containing

Table 1. Solubilization of pellicular Cbl binding proteins

Purified pellicle (11.5 mg protein) was suspended in 5 ml 100 mM-sodium phosphate buffer, pH 7.2, containing 0.1% SDS plus 2 M-urea and incubated at 30 °C for 1 h with gentle stirring. The suspension was then centrifuged at 10000 *g* for 30 min. The supernatant was used as the 0.1% SDS/2 M-urea fraction. The precipitate was resuspended in 5 ml of the same buffer containing 1% SDS, incubated under the above conditions and centrifuged at 10000 *g* for 30 min. The supernatant was used as the 1% SDS fraction.

Fraction	Total protein (mg)	Total activity (pmol Cbl bound)	Specific activity [pmol Cbl bound (mg protein) ⁻¹]	Yield (%)
Pellicle	11.5	58.0	5.0	100
0.1% SDS/2 M-urea	4.0	37.0	9.3	63.8
1% SDS	6.0	18.0	3.0	31.0

glutamate and glucose (both 10 mM). The antibody against pellicular Cbl binding protein (0.1 ml) was added to 0.1 ml of the cell suspension (8.0×10^5 cells) and incubated at room temperature for 2 h. [³H]CN-Cbl was then added to the suspension to 10^{-8} M and the mixture was incubated at 27 °C for 15 s to allow the rapid, initial phase of Cbl uptake and for 2 min to allow the initial and slower secondary phase of uptake to occur. The suspension was immediately filtered through a membrane filter (TM-2P, 24 mm diameter) and washed twice with the above buffer at the same temperature. The radioactivity on the membrane filter was counted using a liquid scintillation counter (Aloka, LSC 903). The Cbl uptake in the secondary phase was calculated by subtracting the radioactivity in the initial phase from that over 2 min.

PAGE. Disc PAGE with 7.5% (w/v) acrylamide at pH 9.4 (Davis, 1964) was done at constant current (2 mA per gel) with bromophenol blue as a migration marker. Proteins were fixed, stained with 10% (v/v) acetic acid and 0.005% Coomassie brilliant blue R 250 in water and destained in 7% (v/v) acetic acid in water. SDS-slab-gel electrophoresis in a linear gradient (7.5–15%, w/v) of polyacrylamide (Laemmli, 1970) was done at constant current (6 mA per gel) with bromophenol blue as a migration marker. Proteins were fixed and stained by incubating the gels successively with: 0.005% Coomassie brilliant blue R 250, 10% acetic acid and 25% (v/v) propan-2-ol in water; then with the same solution containing 10% propan-2-ol instead of 25% propan-2-ol; then with the same solution without propan-2-ol; and then destained in 7% acetic acid in water. Rabbit muscle phosphorylase *b* (*M_r* 94000), bovine serum albumin (BSA, 67000), ovalbumin (43000), bovine erythrocyte carbonic anhydrase (30000), soybean trypsin inhibitor (20100) and bovine milk α -lactalbumin (14400) were used as standard proteins.

For the *M_r* determination of partially purified Cbl binding proteins, the proteins obtained by DEAE-Bio-Gel A chromatography after binding [⁵⁷Co]CN-Cbl by equilibrium dialysis and then treating with 1% SDS at 30 °C for 30 min, were subjected to SDS-slab-gel electrophoresis. The Cbl binding proteins in the gel were detected by autoradiography.

Protein determinations. Protein was measured according to Bradford (1976) using BSA as standard.

Chemicals. [³H]CN-Cbl (5.1 Ci mmol^{-1} , $188.7 \text{ GBq mmol}^{-1}$) and [⁵⁷Co]CN-Cbl ($250 \mu\text{Ci } \mu\text{g}^{-1}$, $9.25 \text{ MBq } \mu\text{g}^{-1}$) were obtained from New England Nuclear and Amersham, respectively. CN-Cbl, 5'-deoxyadenosylcobalamin (Ado-Cbl), methylcobalamin (Me-Cbl) and hydroxocobalamin (OH-Cbl) were obtained from Sigma. Sephacryl S-200 and the electrophoresis calibration kit were obtained from Pharmacia. Freund's complete adjuvant was obtained from Wako. DEAE-Bio-Gel A was obtained from Bio-Rad Laboratories. Membrane filters (TM-2P) were obtained from Toyo Roshi.

RESULTS

Solubilization of pellicular Cbl binding proteins

The Cbl binding protein was partially solubilized from *E. gracilis* pellicle using 0.1% (w/v) SDS/2 M-urea. The residual Cbl binding protein could not be solubilized using this detergent system but could be extracted using 1% (w/v) SDS (Table 1). The results suggest that several Cbl binding proteins occur in the *E. gracilis* pellicle. Other detergents, including Brig 35, Emulgen 910, Lubrol PX, Triton X-100, NR 40, digitonin and deoxycholate, all at 1% (w/v), only partially solubilized the Cbl binding protein (data not shown).

Fig. 1

Fig. 1. DEAE-Bio-Gel A column chromatography of pellicular Cbl binding proteins. The elution profiles of the activity in the protein solubilized with 0.1% SDS/2 M-urea (a) and with 1% SDS (b) are shown. ●, Cbl binding activity; ---, NaCl concentration.

Fig. 2

Fig. 2. PAGE of the purified pellicular Cbl binding protein (10 μ g protein) solubilized with 0.1% SDS/2 M-urea.

DEAE-Bio-Gel A ion exchange chromatography of pellicular Cbl binding proteins

The elution profiles of the DEAE-Bio-Gel A column chromatography of the Cbl binding proteins solubilized by the two detergent systems are shown in Fig. 1 (a, b). The Cbl binding activity in each of the 0.1% SDS/2 M-urea and 1% SDS fractions was eluted as a single peak at about 0.1 M-NaCl, indicating that the two Cbl proteins have a similar surface potential at pH 7.2. Each Cbl binding activity was eluted as a single peak in the void volume on chromatography on Sephacryl S-200. The results suggest that each Cbl binding protein may be aggregated or conjugated with other proteins; however, no information on the aggregation is available.

Some properties of the Cbl binding proteins

When the 0.1% SDS/2 M-urea or 1% SDS fractions were heated at various temperatures for 10 min before determination of the Cbl binding activity, they were stable up to 40 °C and the Cbl binding activity was lost completely at 60 °C. Binding of Cbl to both pellicular binding proteins showed a broad pH dependence. The 0.1% SDS/2 M-urea and 1% SDS fractions showed a maximum activity between pH 3–7 and pH 6–11, respectively. When both proteins were incubated at various pH values for 10 min at 45 °C, the 0.1% SDS/2 M-urea fraction was stable between pH 4 and 8, and at pH 10 still showed 51% of the activity at pH 7, while the 1% SDS fraction was stable between pH 7 and 11, and at pH 3 showed 80% of the activity at pH 7.

Table 2. Purification of pellicular Cbl binding protein

Step	Total protein (mg)	Total activity (pmol Cbl bound)	Specific activity [pmol Cbl bound (mg protein) ⁻¹]	Recovery (%)
Solubilization (0.1% SDS/2 M-urea)	151	475.5	3.1	100
(NH ₄) ₂ SO ₄ fractionation	38.0	364.7	9.6	76.7
DEAE-Bio-Gel A	23.6	342.5	14.5	72.0
Sephacryl S-200	7.7	306.0	39.7	64.4
CM-cellulose	1.6	157.0	98.1	33.0

Metal ions (K⁺, Na⁺, Zn²⁺, Mn²⁺, Mg²⁺, Ca²⁺, Cu²⁺, Ni²⁺, Co²⁺, Fe²⁺ and Fe³⁺, all at 1 mM) had no significant effects and EDTA at 5 mM did not inhibit the activity, indicating that metal ions are not essential for the formation of either binding-protein-Cbl complex. Neither activity was inhibited by the SH-inhibitors *N*-ethylmaleimide, 5,5'-dithiobis(2-nitrobenzoic acid) and mersalyl (all at 1 mM), suggesting that SH-groups are not essential for Cbl binding activity.

Using double reciprocal plots of [³H]CN-Cbl concentration versus the Cbl binding activity of the proteins, the *K_s* values for CN-Cbl of the 0.1% SDS/2 M-urea and 1% SDS fractions were calculated to be 0.22 and 0.19 nM respectively. CN-Cbl binding to the 0.1% SDS/2 M-urea fraction was inhibited 50%, 30% and 50% by OH-Cbl, Me-Cbl and Ado-Cbl (all at 10 nM), respectively, while that to the 1% SDS fraction was inhibited 50%, 42% and 35%, respectively. The results indicate that both pellicular Cbl binding proteins are able to bind these Cbl analogues as well as CN-Cbl. However, neither Cbl binding activity was affected by 2-amino-2-methylpropanolcobalamin (2-AMP-Cbl), an isomer of the alkanolamine group.

The *M_r* values of both Cbl binding protein polypeptides were calculated to be 38 000 by SDS-slab-gel electrophoresis and autoradiography. Only a single CN-Cbl binding polypeptide was detected in each case. The purified proteins had an identical *M_r*.

Purification of the Cbl binding protein solubilized with 0.1% SDS 2 M-urea

This is summarized in Table 2. Disc PAGE of the Cbl binding protein after the final purification step revealed a single protein band (Fig. 2). The Cbl binding protein solubilized with 1% SDS was too labile to be purified to homogeneity.

Effects of antibody on the purified pellicular Cbl binding protein and the purified pellicle

When the purified pellicular Cbl binding protein was pretreated with antibody (0.09 mg protein) against the Cbl binding protein solubilized with 0.1% SDS/2 M-urea at 30°C for 1 h, the Cbl binding activity of the protein was inhibited completely (Fig. 3*a*). However, the Cbl binding activity in the purified *E. gracilis* pellicle was inhibited only 55% by the same pretreatment with the antibody (Fig. 3*b*). The remaining activity in the pellicle treated with the antibody appears to be due to the presence of a Cbl binding protein other than the protein solubilized with 0.1% SDS/2 M-urea. This may be the protein solubilized with 1% SDS, since the antibody did not cross-react with the 1% SDS fraction.

Cbl uptake in the rapid, initial phase was inhibited about 50% by preincubation with the antibody (2.0 mg protein) at 30°C for 1 h, while that in the slower, secondary phase was completely inhibited by the antibody (0.4 mg protein) (Fig. 4). The results strongly suggest that the Cbl binding protein, solubilized with 0.1% SDS/2 M-urea takes part in the slower secondary phase of the Cbl uptake, while the protein solubilized with 1% SDS is probably concerned with the uptake in the rapid initial phase.

Fig. 3

Fig. 4

Fig. 3. Effect of antibody against the protein solubilized with 0.1% SDS/2 M-urea on the Cbl binding activities of the 0.1% SDS/2 M-urea fraction (a) and the purified pellicle (b). The Cbl binding activity was determined after incubating the 0.1% SDS/2 M-urea fraction (40 µg protein) or the purified pellicle (100 µg protein) with the indicated amounts of the antibody at 30 °C for 1 h. Relative activity is the activity as a percentage of that without antibody treatment.

Fig. 4. Effect of the antibody against the protein solubilized with 0.1% SDS/2 M-urea on Cbl uptake by *E. gracilis* cells. Cbl uptake was determined 15 s and 2 min after adding to *E. gracilis* (8.0×10^5 cells) which had been preincubated with the indicated amounts of antibody at room temperature for 2 h. (a) Rapid, initial phase; (b) slower, secondary phase.

DISCUSSION

Varma *et al.* (1961) indicated that the initial binding reaction in Cbl uptake occurred in the cell wall of *E. gracilis* Z. The data of Varma *et al.* (1961) and our finding that purified *E. gracilis* pellicle has a high Cbl binding activity (Table 1) indicate that the Cbl uptake system is embedded in the pellicle. Furthermore, the results of the antibody inhibition experiments indicate that both binding proteins are located in the pellicle.

Using an excellent subcellular fractionation technique, Isegawa *et al.* (1985) showed that Cbl taken up by *E. gracilis* Z is transferred to mitochondria, chloroplasts, microsomes and the cytosol within a few minutes, and that Cbl binding activity occurs in all organelles. Sarhan *et al.* (1980) suggested that the initial binding process of Cbl uptake occurs in the chloroplast envelope in *E. gracilis* Z. However, since *E. gracilis* Cbl binding protein occurs in each organelle, the Cbl binding reaction in the chloroplast itself cannot be considered to take part in the Cbl uptake system.

Of the Cbl binding protein, 65% was solubilized from purified *E. gracilis* pellicle by 0.1% SDS/2 M-urea, and the residual 35% by 1% SDS (Table 1). The outer membrane Cbl receptor of *E. coli* (White *et al.*, 1973) and the intrinsic factor (IF)-Cbl complex receptor (Marcoulliss & Grasbeck, 1977) have been solubilized with Triton X-100 and purified to homogeneity, but Triton X-100 did not solubilize the *E. gracilis* pellicular Cbl binding proteins.

Ca²⁺ is essential for the binding of Cbl to the outer membrane receptor of *E. coli* (White *et al.*, 1973) and that of IF-Cbl complex to its receptor (Cotter *et al.*, 1977). In our experiments, we found that *E. gracilis* pellicular Cbl binding proteins did not require metal ions for the formation of the protein-Cbl complexes and EDTA did not inhibit binding, indicating that the Cbl binding mechanism is different from that of the outer membrane receptor of *E. coli*.

The K_s values of the *E. gracilis* proteins for CN-Cbl (0.22 and 0.19 nM), are similar to those of the outer membrane receptor and the periplasmic Cbl binding protein of *E. coli* (0.3–0.8 nM) (Kenley *et al.*, 1978; Bradbeer *et al.*, 1978). The M_r of both pellicular Cbl binding proteins of *E. gracilis* was 38 000, while those of TC-II (Allen & Majerus, 1972), IF (Allen & Mehlman, 1973), the outer-membrane receptor (Sabet & Schnaitman, 1973) and periplasmic Cbl binding protein (Taylor *et al.*, 1972) of *E. coli* are 60 000, 66 000, 60 000 and 22 000, respectively.

The activity of both *E. gracilis* pellicular Cbl binding proteins was considerably inhibited by three Cbl analogues, OH-Cbl, Ado-Cbl and Me-Cbl, but not by 2-AMP-Cbl, which is an isomer of the alkanolamine group of the Cbl molecule, indicating that the α -coordination position of Cbl, especially the alkanolamine moiety, is more important for the formation of the pellicular binding-protein–Cbl complexes than the β -coordination position. In IF and TC-II in mammals (Hippe *et al.*, 1971) and the outer membrane receptor of *E. coli* (Kenley *et al.*, 1978), the α -coordination position, especially the nucleotide portion, is reported to be essential for the binding of Cbl to the Cbl binding proteins.

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