Purification, Some Properties and Possible Physiological Role of an Extracellular Cobalamin Binding Protein from *Euglena gracilis*

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The extracellular cobalamin (Cbl) binding protein from *Euglena gracilis* was purified and some properties of the protein were studied for the elucidation of its physiological role. The protein was purified about 20-fold with a yield of 15% and was homogeneous on PAGE. SDS-PAGE indicated that the protein had a single type of polypeptide of M_r 56000. The protein could bind some Cbl analogues with different β -coordination moieties, over a wide range of pH values from 4.0 to 9.0, and the K_s value for cyanocobalamin was 1.1 nM. The extracellular Cbl binding protein was located on the cell surface of *E. gracilis*, probably bound in the muciferous layer.

INTRODUCTION

In higher animals, intestinal absorption and subsequent plasma transport of cobalamin (Cbl) is mediated by specific binding proteins, e.g. intrinsic factor (Allen & Mehلمان, 1973) and transcobalamin II (Allen & Majerus, 1972). These binding proteins have been purified and characterized, and their physiological roles have been explained (Herbert, 1959; Rappazzo & Hall, 1972). Heat labile Cbl binding macromolecules have been reported to be formed in the culture broth of some micro-organisms (Ford, 1958), but lack of detailed study on these proteins has limited our understanding of their physiological functions.

An extracellular Cbl binding protein has been reported for *Euglena gracilis* (Kristensen, 1956) and was purified several-fold from the culture broth (Daisley, 1970). Our previous study (Watanabe *et al.*, 1987) has shown that *E. gracilis* Cbl binding proteins are distributed in the cytosol, mitochondria and microsomes, and that the extracellular Cbl binding protein is immunologically identical to the cytosolic one. In the present work we describe the extensive purification of the extracellular Cbl binding protein and discuss the possible physiological role of this protein.

METHODS

Organism and culture. *E. gracilis* SM-ZK, a streptomycin-bleached mutant of *E. gracilis* Z, was cultured for 5 d at 27 °C and 2000 lx in a Cbl-limited (0.05 $\mu\text{g l}^{-1}$) Koren–Hutner medium (Koren & Hutner, 1967).

Cbl binding assay. This was done as described previously (Watanabe *et al.*, 1988).

Purification of extracellular Cbl binding protein. All purification procedures were done at 0–4 °C. A 5 d *E. gracilis* culture (15 l) was centrifuged at 2000 g for 5 min to remove the cells. *o*-Phenanthroline (5 mM) an *E. gracilis* extracellular proteinase inhibitor, and Hyflo super-cel (200 g) were added to the supernatant. The suspension was filtered to completely remove *E. gracilis* cells and the filtrate was used as a crude preparation of extracellular Cbl binding protein. The filtrate (14.7 l) was concentrated to a final volume of 1.75 l using polyethylene glycol (PEG) 20000, and dialysed overnight against 16 litres 10 mM-potassium phosphate buffer, pH 7.2. The dialysed solution was applied to a column (2 $\times$ 10 cm) of DEAE-cellulose equilibrated with 10 mM-potassium phosphate buffer,

Abbreviations: Cbl, cobalamin; CN-Cbl, cyanocobalamin; OH-Cbl, hydroxocobalamin; Me-Cbl, methylcobalamin; Ado-Cbl, 5'-deoxyadenosylcobalamin; 2-AMP-Cbl, 2-amino-2-methylpropanolylcobalamin.

pH 7.2. The column was washed with 100 ml of the same buffer and eluted with 400 ml of a linear (0–0.5 M) KCl gradient in the same buffer. The active fractions (178 ml) were combined and dialysed overnight against 3 l of the same buffer. The dialysed solution was applied to a column (1 × 5 cm) of DEAE-cellulose equilibrated with 10 mM-potassium phosphate buffer, pH 7.2, and eluted with 200 ml of a linear (0–0.4 M) KCl gradient in this buffer. The active fractions were combined and concentrated to a final volume of 2 ml by use of PEG 20 000. The concentrated solution was applied to a column (1.5 × 90 cm) of Sephacryl S-300 equilibrated with 100 mM-potassium phosphate buffer, pH 7.2, and eluted with the same buffer. The active fractions were collected, concentrated to a final volume of 1 ml by use of PEG 20000 and stored at –20 °C until use.

PAGE. SDS-PAGE was done under the same conditions and using the same standards as described in Watanabe *et al.* (1988).

Western blotting. Electrophoretic transfer to a nitrocellulose sheet (TM-2, 0.45 µm) was done at 50 V for 90 min at 0 °C as described by Burnette (1981). *E. gracilis* extracellular Cbl binding protein on the nitrocellulose sheet was detected immunologically with antibody against an *E. gracilis* cytosolic Cbl binding protein (Watanabe *et al.*, 1987) and horseradish-peroxidase-anti-rabbit IgG goat serum conjugate as described by Towbin *et al.* (1979).

Properties of the protein. The pH dependency was determined by using citrate buffer for pH 2.0–5.0, Tris/acetate buffer for pH 5.0–9.0, and glycine/NaOH buffer for pH 9.0–11.0, all at 100 mM, as the dialysis buffers. The pH stability was determined by using the protein pre-treated at various pH values for 10 min at 45 °C. The thermal stability was determined by using the protein pre-incubated at various temperatures between 0 and 70 °C for 10 min at pH 7.2. The K_d value for cyanocobalamin (CN-Cbl) was calculated from double reciprocal plots of the amount of Cbl bound to the protein versus the ligand concentration (0.2–20 nM). For determination of inhibition of CN-Cbl binding activity by some Cbl analogues, hydroxocobalamin (OH-Cbl), methylcobalamin (Me-Cbl), 5'-deoxyadenosylcobalamin (Ado-Cbl) (all at 10 nM) and 60 nM-2-amino-2-methylpropanolylcobalamin (2-AMP-Cbl) were added into the dialysis buffer containing 10 nM-[³H]CN-Cbl. For experiments on effects of thiol-reagents [mersalyl acid, 5,5'-dithio-bis(2-nitrobenzoic acid), *N*-ethylmaleimide, iodoacetamide, dithiothreitol, 2-mercaptoethanol and reduced glutathione] and the chelater EDTA, each reagent, at 1 mM, was added to the dialysis bag and buffer at 1 mM. All experiments were done with the purified Cbl binding protein (2–4 µg protein). All results presented are mean values of three experiments.

Inhibition of Cbl uptake by the antibody against *E. gracilis* cytosolic Cbl binding protein. *E. gracilis* grown for 5 d in Cbl-limited medium was centrifuged at 2000 *g* for 5 min to collect cells, which were washed twice with 10 mM-sodium phosphate buffer, pH 7.2, containing 0.9% (w/v) NaCl, suspended in the same buffer containing glutamate and glucose (both 10 mM) and incubated with shaking at 27 °C for 3 h. The *E. gracilis* cells (10⁶ cells) were pre-treated with the antibody against *E. gracilis* cytosolic binding protein at 27 °C for 2 h. The Cbl uptake was started by adding [³H]CN-Cbl (9.2 Ci mmol⁻¹, 340 GBq mmol⁻¹) at 10 nM to the pre-treated *E. gracilis* cell suspension, allowed to proceed for 15 s or for 2 min at 27 °C and stopped by filtering through a membrane filter (TM-2P). The membrane was washed twice with 10 mM-sodium phosphate buffer, pH 7.2, containing 0.9% NaCl at 27 °C. The membrane filters were dried and counted with a liquid scintillation counter (Aloka, LSC 903).

Protein determination. Protein was determined according to Bradford (1976) using BSA as a standard.

Chemicals. [³H]CN-Cbl (9.2 Ci mmol⁻¹, 340 GBq mmol⁻¹) was obtained from New England Nuclear. CN-Cbl, OH-Cbl, Ado-Cbl, Me-Cbl and horseradish peroxidase-anti-rabbit IgG goat serum conjugate were obtained from Sigma. Sephacryl S-300 and the electrophoresis calibration kit were obtained from Pharmacia. Membrane filters (TM-2 and TM-2P) were purchased from Toyo Roshi.

RESULTS AND DISCUSSION

Extracellular Cbl binding activity during growth

The Cbl binding activity reached a maximum [0.18 pmol (ml medium)⁻¹] in the early stationary phase and remained constant thereafter up to 9 d (Fig. 1). The results indicate that the extracellular Cbl binding protein is not secreted into the culture medium until *E. gracilis* cells reach the stationary phase.

Purification of the extracellular Cbl binding protein

This was done as described in Methods; the results are summarized in Table 1. The Cbl binding protein was purified about 20-fold with a yield of 15% from the culture medium of *E. gracilis* grown for 5 d. The purified Cbl binding protein showed a specific activity of 936.6 pmol (mg protein)⁻¹, which was more than 20 times as pure as the preparation reported by Daisley (1970). Disc PAGE in 7.5% (w/v) acrylamide at pH 9.4 of the purified protein revealed a single band.

Fig. 1. Extracellular Cbl binding activity during *E. gracilis* growth. *E. gracilis* cells (23.5×10^6 cells), which were pre-cultured for 5 d in Koren–Hutner medium, were transferred to Cbl-limited medium (150 ml). Samples (1 ml) were centrifuged (6000 *g*, 5 min) and the supernatant was used as a crude extracellular Cbl binding protein preparation. The data represent the mean of values from four experiments. ●, Cbl binding activity; ○, cell number.

Table 1. *Purification of the extracellular Cbl binding protein from an E. gracilis culture*

The data are typical of purification data from five experiments.

Step	Volume (ml)	Protein (mg)	Total activity (pmol Cbl bound)	Specific activity [pmol Cbl bound (mg protein) ⁻¹]	Recovery (%)
Culture medium	14700	104.7	4789.9	45.7	100
PEG 20000	1750	70.0	4585.1	65.5	95.8
First DEAE-cellulose	178	10.7	2561.3	239.8	53.5
Second DEAE-cellulose	2	2.5	1354.5	541.8	28.3
Sephacryl S-300	1	0.8	749.3	936.6	15.6

Some properties of the purified extracellular Cbl binding protein

The pH dependency for the binding of Cbl to the protein, and the pH stability, were very broad (pH 4.0–9.0). Thiol-groups and metal ions were apparently not involved in Cbl binding since it was not affected by thiol inhibitors or EDTA. The inhibition of CN-Cbl binding activity by the Cbl analogues OH-Cbl, Me-Cbl and Ado-Cbl (45%, 35% and 46% inhibition, respectively) but not by 2-AMP-Cbl, suggests that the α -coordination moiety of the Cbl molecule is more important for formation of the protein-Cbl complex than the β -coordination moiety. These properties were similar to those of the cytosolic and pellicular Cbl binding proteins, but the extracellular Cbl binding protein was stable up to 30 °C and therefore showed a lower thermal stability than these Cbl binding proteins (Watanabe *et al.*, 1987, 1988). The extracellular Cbl binding protein showed a lower affinity for CN-Cbl ($K_s = 1.1$ nM) than did the pellicular binding proteins (Watanabe *et al.*, 1988), but had a similar affinity to the cytosolic binding proteins ($K_s = 1$ –2 nM) (Watanabe *et al.*, 1987). The M_r of the polypeptide of the extracellular Cbl binding protein was estimated to be 56 000 by SDS-PAGE, while those of the cytosolic and pellicular proteins were 66 000 and 38 000, respectively (Watanabe *et al.*, 1987, 1988). The native extracellular Cbl binding protein was eluted in the void fraction on Sephacryl S-300 gel filtration, indicating that the protein has an M_r of more than 1 500 000, unless non-specific aggregation occurs. The M_r of the native protein could not be determined accurately since there is no information available on aggregation. The cytosolic and pellicular Cbl binding proteins showed identical behaviour on gel filtration (Watanabe *et al.*, 1987, 1988).

Possible location of the extracellular Cbl binding protein

The extracellular Cbl binding protein is immunologically identical to the cytosolic binding proteins (Watanabe *et al.*, 1987). In Western blotting, after SDS-PAGE of the crude extracellular Cbl binding protein, no proteins other than that with an M_r of 56 000 were detected when immunoreacted with the antibody against the cytosolic Cbl binding protein. Moreover, *E.*

Fig. 2. Aggregation of *E. gracilis* cells by antibody against the cytosolic Cbl binding protein. *E. gracilis* cells were pre-treated with either unimmunized-rabbit serum (a) or antibody against the cytosolic Cbl binding protein (b). Bars, 100 μ m.

gracilis cells, when treated with the antibody, aggregated (Fig. 2). The ability of the antibody to agglutinate the cells provides evidence that the extracellular Cbl binding protein occurs as a surface component, probably embedded in the muciferous layer which surrounds the *E. gracilis* cell. These results suggest that the extracellular Cbl binding protein is not due to the leakage of intracellular Cbl binding proteins as a result of cell destruction but that the cytosolic Cbl binding protein, after some processing, is secreted and linked to the muciferous layer. The destruction of the muciferous layer by collision among *E. gracilis* cells in the stationary phase may cause the leakage of the extracellular Cbl binding protein into the culture medium.

Physiological role of the extracellular Cbl binding protein

The kinetics of Cbl uptake by *E. gracilis* have been reported to be biphasic (Sarhan *et al.*, 1980), and we have shown that *E. gracilis* pellicular Cbl binding proteins take part in the biphasic uptake system (Watanabe *et al.*, 1988). The extracellular Cbl binding protein, which is probably located on the cell surface, is not considered to be involved in this biphasic process since neither the Cbl uptake in the initial rapid phase nor that in the slower secondary phase were inhibited by treating the cells with the antibody against the cytosolic Cbl binding protein. The results indicate that the extracellular Cbl binding protein functions as an accumulator of Cbl in the cell surface region. The evidence presented suggests that *E. gracilis* firstly binds the trace amount of Cbl in the environment using the extracellular Cbl binding protein; the subsequent uptake of the accumulated Cbl in the cell surface region would also involve the pellicular Cbl binding proteins which act as the Cbl carrier proteins in the *E. gracilis* cell membrane.

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