Purification and some properties of cytosolic cobalamin-binding protein in *Euglena gracilis*

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In *Euglena gracilis* SM-ZK, a bleached mutant of *E. gracilis* z, the cobalamin- (Cbl-)binding activity was distributed in cytosol (49.2%), mitochondria (20.3%) and microsomal fraction (20.4%). The cytosolic Cbl-binding activity gave two major peaks in isoelectric focusing. The Cbl-binding protein with pI 3.2 was purified 6500-fold in a yield of 19.9%, and that with pI 4.7 5800-fold in a yield of 11.9%. The monomeric M_r values of both Cbl-binding proteins were about 66000. The Cbl-binding activity of both proteins showed a very low pH-dependency, and thiol groups and metal ions were not concerned with the Cbl-binding activities. The K_s values of the Cbl-binding proteins with pI 3.8 and 4.7 for CN-Cbl were 1.0 and 2.0 nM respectively. The Cbl-binding protein with pI 3.8 was shown to be immunologically identical with the protein with pI 4.7 by double-immunodiffusion experiments against antibody to the protein with pI 3.8. The two cytosolic Cbl-binding proteins did not show the activities of Cbl-dependent enzymes in *E. gracilis*, N^5 -methyltetrahydrofolate:homocysteine methyltransferase, methylmalonyl-CoA mutase and ribonucleotide reductase, suggesting that the two cytosolic Cbl-binding proteins play a physiological role as intracellular Cbl carriers.

INTRODUCTION

Cbl is synthesized only in some micro-organisms. In the human, ingested Cbl is bound by the gastric intrinsic factor (IF) [1], which facilitates Cbl absorption in the distal intestine [2,3]. The subsequent plasma transport of Cbl is mediated by transcobalamin-II (TC-II) [4,5], which delivers Cbl to various tissues [6]. Several studies have suggested that the TC-II-Cbl complex is bound to a cell-surface receptor [7] and internalized by endocytosis [8]. The protein moiety is degraded after the endocytotic vesicles have fused with lysosomes, releasing free Cbl into the cytosol [9]. It is obscure, however, how the cytosolic Cbl is transported to organelles and transformed into coenzyme forms (Ado-Cbl or Me-Cbl).

Euglena gracilis, having well-developed organelles, like higher animals, requires Cbl for growth [10] and readily gives Cbl-limited cells. Accordingly, for elucidation of the intracellular transport system and physiological function of Cbl, this protozoan is a very useful organism as a model of higher-animal systems. In the present work we report the subcellular distribution of the Cbl-binding proteins in *E. gracilis*. We have also purified two Cbl-binding proteins from *Euglena* cytosol, and describe some properties of the proteins, with discussion on their physiological roles.

EXPERIMENTAL

Organism and culturing

E. gracilis SM-ZK, a streptomycin-bleached mutant of *E. gracilis* z, was grown for 5 days at 27 °C under illumination (2000 lx) in the Cbl-limited (0.05 µg/l) Koren-Hutner medium [11].

Subcellular fractionation

A cell homogenate of *E. gracilis* was obtained by partial tryptic digestion of the pellicle followed by mild mechanical disruption and fractionation by differential centrifugation in accordance with Tokunaga *et al.* [12].

Cbl-binding assay

The Cbl-binding activity was assayed by the equilibrium-dialysis method. A dialysis bag containing 100 µl of a sample obtained by the above method was equilibrated with 10 ml of 10 mM-potassium phosphate buffer, pH 7.2, containing 10 nM-CN-[G-³H]Cbl and 0.0002% KCN at 4 °C for 24 h. CN-[G-³H]Cbl bound to the sample was determined by using a liquid-scintillation counter (Aloka model 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 bag.

Assay of some marker and Cbl-dependent enzymes

Isocitrate dehydrogenase, a mitochondrial marker enzyme, and glutamate dehydrogenase, a cytosol marker enzyme in *Euglena*, were assayed by the methods of Oda *et al.* [13] and Tokunaga *et al.* [12] respectively. Glucose-6-phosphatase, a microsomal marker enzyme, was assayed by the method of de Duve *et al.* [14].

N^5 -Methyltetrahydrofolate:homocysteine methyltransferase [15], methylmalonyl-CoA mutase [16] and ribonucleotide reductase [17] were assayed by the methods described in the cited references.

Purification of cytosolic Cbl-binding protein

All purification procedures were performed at 0–4 °C. Partial tryptic digestion of *Euglena* cells (50 g wet wt.)

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

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was employed to obtain a cell homogenate. The homogenate was centrifuged at 100 000 *g* for 60 min, and the supernatant was used as a cytosolic fraction. The cytosolic fraction (500 ml) was applied on to a DEAE-cellulose column (3 cm × 45 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.2, at a flow rate of 24 ml/h. The column was washed with 200 ml of the same buffer and then eluted with 1 litre of a linear gradient of 0–0.4 M-KCl in the same buffer. The active fractions were combined and adjusted to 70 % saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was collected by centrifugation at 10 000 *g* for 1 h and then dissolved in 5 ml of 100 mM-potassium phosphate buffer, pH 7.2. After dialysis overnight against 1 litre of the same buffer, the solution was applied on to a column (3 cm × 100 cm) of Sephacryl S-300 (superfine grade) equilibrated with 100 mM-potassium phosphate buffer, pH 7.2, and eluted with the same buffer at a flow rate of 20 ml/h. The active fractions were combined and concentrated to a final volume of 2 ml by using poly(ethylene glycol) 20 000. The concentrated solution was subjected to isoelectric focusing. The active fractions, after being collected and concentrated to a final volume of 1 ml, were applied on to a Sephadex G-25 column (1.5 cm × 40 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.2, to remove Pharmalyte and eluted with the same buffer. The active fractions were combined, concentrated to a final volume of 1 ml by using poly(ethylene glycol) 20 000 and stored at –20 °C.

Preparation of the antibody against the cytosolic Cbl-binding protein with pI 3.8

The purified cytosolic Cbl-binding protein with pI 3.8 (50 µg of protein), which was suspended in 1.0 ml of 10 mM-sodium phosphate buffer, pH 7.2, containing 0.15 M-NaCl, was emulsified with 1.0 ml of Freund's complete adjuvant. A female rabbit (about 2 kg) was hypodermically injected with 2.0 ml of the emulsion at multiple sites. At 2 weeks after the injection, 50 µg of the cytosolic Cbl-binding protein was injected in the same manner as described above. A booster injection of 50 µg of the protein in 1.0 ml of Freund's incomplete adjuvant was given 2 weeks later at the same sites. Then 2 days after the booster injection the rabbit was bled and serum was prepared by the standard method. The serum was applied on to a column of DEAE-cellulose equilibrated with 20 mM-sodium phosphate buffer, pH 8.0, and eluted with the same buffer. The IgG fractions were combined and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation, and the precipitate was dissolved in 10 mM-sodium phosphate buffer, pH 7.2, containing 0.15 M-NaCl and dialysed against the same buffer.

Preparation of extracellular Cbl-binding protein

A 5-day *Euglena* culture (3 litres) was centrifuged at 2000 *g* for 5 min to remove the cells, and to the supernatant was added 40 g of Hyflo Super-Cel, a filter powder. The suspension was filtered to remove contaminated *Euglena* cells. The filtrate was used as a crude extracellular Cbl-binding protein, and concentrated to a final volume of 33 ml by using poly(ethylene glycol) 20 000. The concentrated solution, after dialysis overnight against 8 litres of 10 mM-potassium phosphate buffer, pH 7.2, was applied on to a column (1.5 cm × 10 cm) of DEAE-cellulose equilibrated with the same buffer. The column was washed with 100 ml of the same buffer and

Table 1. Distribution of activities of Cbl-binding protein and marker enzymes in subcellular fractions of *E. gracilis*

Detailed procedures of subcellular fractionation of *E. gracilis* are described in the Experimental section. The activities of Cbl-binding protein and marker enzymes were determined by the methods described in the Experimental section. The marker enzymes, glutamate dehydrogenase, isocitrate dehydrogenase, and glucose-6-phosphatase, showed activities of 2.88, 0.89 and 0.32 µmol/min respectively in the crude homogenates. Specific Cbl-binding activities (pmol/mg of protein) are given in parentheses. The values presented are typical of the distribution pattern obtained in four experiments.

	Enzyme activity (% of activity in crude extract)		
	Cytosol	Mitochondria	Microsomes
Cbl-binding activity	49.2 (5.30)	20.3 (3.15)	20.4 (6.90)
Glutamate dehydrogenase	94.1	0	0
Isocitrate dehydrogenase	0	86.9	0
Glucose-6-phosphatase	0	0	72.3

eluted with 300 ml of a linear gradient of 0–0.4 M-KCl in the same buffer. The active fractions were combined and concentrated to a final volume of 3.0 ml by using poly(ethylene glycol) 20 000. The extracellular Cbl-binding protein thus obtained was purified about 5-fold in a yield of 53.4 % over the *Euglena* culture medium. This preparation showed a specific activity of 1.18 nmol/mg of protein.

Isoelectric focusing

Isoelectric focusing was performed on a 110 ml column. The carrier ampholytes (Pharmalyte) of pH 2.5–5.0 and 3.0–10.0 were mixed in equal proportions and used at 1 %, and the density gradients were made with 0–40 % (w/v) sucrose. The temperature was 2 °C, the potential 400 V and the running time 56 h.

Inhibition of the Cbl-binding activity by lectins

The purified Cbl-binding proteins (0.04 µg of protein) were preincubated for 6 h at 4 °C with concanavalin A and with *Dolichos biflorus* lectin (0–5 µg of protein). The Cbl-binding activity of the treated proteins was assayed by the equilibrium-dialysis method.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed in 7.5–15 %-linear-gradient polyacrylamide slab gels as described by Laemmli [18]. Electrophoresis was carried out at a constant current (6 mA/gel), with Bromophenol Blue as a migration marker. Proteins in the gel were stained with Coomassie Brilliant Blue R-250 and destained in 7 % (v/v) acetic acid. Rabbit muscle phosphorylase b (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), bovine erythrocyte carbonic anhydrase (M_r 30 000), soya-bean trypsin inhibitor (M_r 20 000) and bovine milk α-lactalbumin (M_r 14 000) were used as standard proteins.

Table 2. Purification of cytosolic Cbl-binding proteins from *Euglena* cytosol

Details of the purification are described in the Experimental section.

	Volume (ml)	Protein (mg)	Total activity (nmol)	Specific activity (nmol/mg of protein)	Recovery (%)
Cytosolic fraction	500	975	2.985	0.003	100
DEAE-cellulose	120	78.0	2.115	0.027	70.9
Sephacryl S-300	3	0.06	1.155	19.25	38.7
Isoelectric focusing					
pI 3.8	1	0.03	0.595	19.83	19.9
pI 4.7	1	0.02	0.355	17.75	11.9

Electrophoretic transfer

The transfer procedures were performed by the Western blotting method as described by Burnette [19]. TM-2, 0.45 μ m pore size (Toyo Roshi Co., Osaka, Japan), was used as the nitrocellulose sheet. Electrophoresis was accomplished at 50 V for 90 min at 0 °C. The *Euglena* cytosolic Cbl-binding proteins on the nitrocellulose sheet were detected immunologically with the antibody against the cytosolic Cbl-binding protein and horseradish-peroxidase-conjugated goat anti-(rabbit IgG) serum as described by Towbin *et al.* [20].

Double immunodiffusion

The double-immunodiffusion [21] experiments were performed in Ouchterplates (Eiken Chemical Co., Tokyo, Japan).

Protein determination

Protein was measured by the method of Bradford [22], with bovine serum albumin as the standard.

Materials

N^5 -Tetrahydro[Me- 14 C]folate (7.7 Ci/mmol) and [3 H]CTP (11.8 Ci/mmol) were obtained from New England Nuclear. CN-[G- 3 H]Cbl (5.1 Ci/mmol) was made to order by New England Nuclear. DL-Methyl[3- 14 C]malonyl-CoA was prepared enzymically by the method of Cannata *et al.* [23]. CN-Cbl, Ado-Cbl, Me-Cbl and OH-Cbl were obtained from Sigma Chemical Co. Freund's complete and incomplete adjuvants were obtained from Wako Pure Chemical Industries, Osaka, Japan. Concanavalin A, *Dolios diflorus* lectin and horseradish-peroxidase-conjugated goat anti-(rabbit IgG) serum were obtained from Sigma Chemical Co. Sephacryl S-300, Pharmalyte and an electrophoresis calibration kit were obtained from Pharmacia Fine Chemicals.

RESULTS

Distribution of the Cbl-binding activity

Table 1 shows distribution of the Cbl-binding activity and the activities of marker enzymes in the subcellular fractions from differential centrifugation of a homogenate of *E. gracilis*. The Cbl-binding activity was distributed 49.2 %, 20.3 % and 20.4 % in the cytosolic, mitochondrial and microsomal fractions respectively. The Cbl-binding activities in the mitochondrial and microsomal fractions were not due to contamination of

the cytosolic fraction, since washing these fractions with 10 mM-Mops/KOH buffer, pH 7.5, containing 0.25 M-sucrose did not remove the activity. The results indicate that the Cbl-binding activity is distributed in each organelle of *E. gracilis* and that the cytosolic fraction contains about half of the total *Euglena* Cbl-binding activity.

Purification of the cytosolic Cbl-binding protein

Purification of the cytosolic Cbl-binding protein of *E. gracilis* was performed as described in the Experimental

Fig. 1. Isoelectric-focusing chromatography of cytosolic Cbl-binding proteins

Isoelectric focusing was performed as described in the Experimental section. A 3 ml portion of the Cbl-binding protein (0.06 mg of protein) from the Sephacryl S-300 step was subjected to isoelectric focusing. Fractions (2.0 ml) were collected from the bottom of the column, and the pH of each fraction was measured at 2 °C by means of a model 225 pH/ion-meter (Iwaki Glass). The Cbl-binding activity was assayed as described in the Experimental section. ●, Cbl-binding activity; ○, pH.

Table 3. Summary of some properties of *Euglena* cytosolic Cbl-binding proteins

The pH-dependency was determined by using sodium 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, at 100 mM for the dialysis buffer. The pH-stability was determined by using the proteins pretreated at the various pH values described above for 10 min at 45 °C. The thermal stability was determined by using the proteins preincubated at various temperatures between 20 and 80 °C for 10 min at pH 7.2. The K_s values for CN-Cbl were calculated from double-reciprocal plots of the amount of Cbl bound to the proteins and the ligand concentration (0.2–20 nM). For experiments on the inhibition of CN-Cbl-binding activities by certain Cbl analogues, OH-Cbl, Me-Cbl, Ado-Cbl (each at 10 nM) and 2-AMP-Cbl (60 nM) were included in the dialysis buffer containing 10 nM-CN-[G-³H]Cbl. For experiments on the effects of thiol-group reagents and chelator, 5,5'-dithiobis-(2-nitrobenzoic acid), *N*-ethylmaleimide, dithiothreitol, 2-mercaptoethanol and EDTA at 1 mM were used, and each reagent was included in the dialysis bag and buffer. All experiments described above were performed with the Cbl-binding proteins (0.04 µg of protein) obtained by the final purification step. The Cbl-binding activity was assayed by the equilibrium-dialysis method described in the Experimental section. M_r determination by SDS/polyacrylamide-gel electrophoresis was performed as described in Fig. 4 legend.

	Peak 1 protein	Peak 2 protein
pH-dependency	Low (4.0–10.0)	Low (6.0–9.0)
pH-stability	pH 4.0–7.5	pH 4.0–7.5
Thermal stability	< 40 °C	< 50 °C
K_s value for CN-Cbl	1.0 nM	2.0 nM
M_r (monomer)	66000	66000
Inhibition of CN-Cbl-binding activity by some Cbl analogues		
OH-Cbl	20 %	36 %
Me-Cbl	15 %	36 %
Ado-Cbl	54 %	63 %
2-AMP-Cbl	0 %	0 %
Inhibitions		
Thiol-group inhibitor	Insensitive	Insensitive
Chelator	Insensitive	Insensitive

section. The results are summarized in Table 2. The cytosolic Cbl-binding activity was obtained as two major peaks in the acidic region by isoelectric focusing, the final purification step (Fig. 1). The first peak (peak 1 protein) and the second peak (peak 2 protein) were located at pH 3.8 and 4.7 respectively. The peak 1 protein was purified about 6500-fold in a yield of 19.9 % and the peak 2 protein about 5800-fold in 11.9 % yield over the *Euglena* cytosol. Polyacrylamide-disc-gel electrophoresis in 7.5 % polyacrylamide at pH 9.4 of each protein of the final purification step revealed a single protein band.

Some properties of the cytosolic Cbl-binding proteins

Some properties of the purified cytosolic Cbl-binding proteins are summarized in Table 3. When the cytosolic Cbl-binding proteins were kept at various temperatures for 10 min, the peak 1 protein was stable up to 40 °C and completely inactivated at 50 °C, whereas the peak 2 protein was stable up to 50 °C and inactivated at 60 °C.

Fig. 2. Effect of concanavalin A and *Dolichos biflorus* lectin on the Cbl-binding activity

The Cbl-binding activity of the proteins (0.04 µg of protein) treated by the lectins was assayed as described in the Experimental section. The specific activities of peak 1 and peak 2 proteins at pH 7.2 were 19.8 and 17.5 nmol/mg of protein respectively. Relative activity is expressed as percentage of the activity with no addition of the lectins. The data are represented by the mean of values obtained from three experiments. ○, Peak 1 protein; ●, peak 2 protein.

The Cbl-binding proteins did not show sharp pH-dependency for their activities. The pH optima of the Cbl-binding activities of the peak 1 and peak 2 proteins were 4.0–10.0 and 6.0–9.0 respectively. Both the proteins, when pretreated at various pH values for 10 min at 45 °C, were stable between pH 4.0 and 7.5, but inactivated above pH 7.5. The K_s values of the peak 1 and peak 2 proteins for CN-Cbl were 1.0 nM and 2.0 nM respectively. The cytosolic Cbl-binding proteins were scarcely inhibited by such thiol-group inhibitors as 5,5'-dithiobis-(2-nitrobenzoic acid) and *N*-ethylmaleimide at 1 mM and were not activated by dithiothreitol and 2-mercaptoethanol at 1 mM. The results suggest that thiol groups are not involved in the active centre of the Cbl-binding proteins. Various metal ions (Na⁺, Mg²⁺, Ca²⁺, Zn²⁺, Ni²⁺, Co²⁺, Fe²⁺ and Fe³⁺, all at 1 mM) and 1 mM-EDTA did not affect either of the Cbl-binding activities. The binding of CN-Cbl to the peak 1 protein was inhibited by 54 %, 15 % and 20 % by Ado-Cbl, Me-Cbl and OH-Cbl at 10 nM respectively, and that to the peak 2 protein was inhibited by 63 %, 36 % and 36 % by Ado-Cbl, Me-Cbl and OH-Cbl respectively. The results suggest that both peak 1 and peak 2 proteins bind specifically Ado-Cbl and CN-Cbl. However, the CN-Cbl-binding activities of both proteins were not inhibited by 60 nM-2-AMP-Cbl, an isomer with modified alkanolamine group, suggesting that the α -co-ordination position of the Cbl molecule, especially for an alkanolamine group, is more important

Fig. 3. Immunodiffusion study of the cytosolic Cbl-binding proteins

The Ouchterlony agar plate was incubated at 4 °C for 24 h after the antibody against the peak 1 protein (20 µg of protein) and the indicated samples (20–50 µg of protein) were injected in the wells. The agar plate was washed with 10 mM-sodium phosphate buffer, pH 7.2, containing 0.15 M-NaCl to remove proteins other than the immunoprecipitins. The precipitins in agar plate were stained with Amide Black 10B and destained in 5% acetic acid. Wells 1 and 4, peak 1 protein, antigen; well 2, peak 2 protein; wells 3, and 5, extracellular Cbl-binding protein; well 6, mitochondrial fraction; centre well A, antibody against the peak 1 protein.

for the formation of cytosolic protein–Cbl complex than is the β -co-ordination position.

Effect of lectin on the Cbl-binding activity

The inhibition of both Cbl-binding activities by concanavalin A and *Dolichos biflorus* lectin is shown in Fig. 2. Concanavalin A and *Dolichos biflorus* lectin are known to bind specifically α -D-mannoside and α -D-galactosaminide respectively. Concanavalin A hardly inhibited the activity of the peak 1 protein, but the lectin inhibited 25% of the Cbl-binding activity of the peak 2 protein, indicating that the latter protein includes some sugar chain probably containing an α -mannoside linkage in the vicinity of the Cbl-binding site. The peak 1 and peak 2 proteins were not inhibited by *Dolichos biflorus* lectin. It seems that neither protein contains a galactoside structure near the active sites. The sugar composition of the proteins could not be analysed because the contents in the purified proteins were too low.

Immunodiffusion study

As shown in Fig. 3, a fusion was formed at the junction of the precipitin lines between the peak 1 and 2 proteins and the anti-(peak 1 protein) serum, suggesting that the peak 1 protein is immunologically identical with the peak 2 protein and that the different pI values of both

Fig. 4. Determination of M_r values of Cbl-binding proteins by SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis and Western blotting were performed as described in the Experimental section. The purified cytosolic Cbl-binding proteins (each 5 µg of protein) were subjected to electrophoresis in slab gel and transferred to a nitrocellulose sheet by Western blotting. Lane 1, peak 1 protein; lane 2, peak 2 protein.

proteins are due to microheterogeneity. Immunoprecipitin lines were also formed against *Euglena* extracellular Cbl-binding protein and a mitochondrial fraction. The precipitin lines against the extracellular protein and the peak 1 and peak 2 proteins were fused. The results show that the extracellular Cbl-binding protein is derived from the cytosolic proteins. Spurs were formed at the junction of the precipitin lines between the mitochondrial fraction and the extracellular and/or the cytosolic Cbl-binding proteins against the antiserum, indicating that the extracellular and the cytosolic binding proteins are immunologically different from the mitochondrial one, but that these Cbl-binding proteins have common antigen determinants.

M_r determination

The native peak 1 and peak 2 proteins were eluted in the void fractions of a Sephacryl S-300 column (1 cm × 90 cm) equilibrated with 100 mM-potassium phosphate buffer, pH 7.2. The results indicate that both proteins have an M_r of more than 1 500 000 unless non-specific aggregation occurs. The accurate M_r value of the native proteins could not be determined because of lack of information concerning aggregation.

Fig. 4 shows the electrophoretic patterns of the two purified cytosolic Cbl-binding proteins after they had been transferred to a nitrocellulose sheet by Western blotting. The peak 1 and peak 2 proteins showed the same mobility in the electrophoresis. The monomeric M_r of both Cbl-binding proteins was calculated to be about 66 000.

Cbl-dependent enzyme activity

The Cbl-dependent enzymes N^5 -methyltetrahydrofolate:homocysteine methyltransferase, methylmalonyl-CoA mutase and ribonucleotide reductase are known to occur in *E. gracilis*. These enzyme activities were determined in the purified cytosolic Cbl-binding proteins. These enzyme activities were not detected at all, confirming that the cytosolic Cbl-binding proteins do not function as the Cbl-dependent enzymes.

DISCUSSION

Daisley [24] has isolated Cbl-binding protein from a cell homogenate of *E. gracilis*. Although the protein was purified about 10-fold over the cell homogenate, it was not homogeneous. The Cbl-binding protein was distributed in all organelles of *E. gracilis* in our experiments (Table 1). Therefore it is not certain where the Cbl-binding protein isolated by Daisley was located in *Euglena* cell.

We isolated homogeneously the Cbl-binding protein from *Euglena* cytosol for the purpose of elucidating its physiological function. The Cbl-binding activity gave two major peaks separable by isoelectric focusing (Fig. 1). The pI of the peak 1 protein was 3.8 and that of the peak 2 protein 4.7. The pI values of the cytosolic Cbl-binding proteins resemble those of IF TC-II and R-binder [25,26]. The different pI values of the two cytosolic Cbl-binding proteins were shown to be due to microheterogeneity by the immunodiffusion study (Fig. 3). IF, TC-II and R-binder have been reported to show the microheterogeneity by isoelectric focusing [25–27]. The monomeric M_r values of both *Euglena* cytosolic Cbl-binding proteins were 66 000 identically, whereas those of TC-II and IF are 60 000 [4] and 59 000 [1] respectively. Nevertheless, the *Euglena* cytosolic Cbl-binding proteins were eluted in the void fractions of Sephacryl S-300 gel filtration. Daisley [24] also has obtained similar results from Sephadex G-200 gel filtration. These observations suggest that the cytosolic Cbl-binding proteins are aggregated with each other and alternatively have an M_r of more than 1 500 000. Detailed information on the aggregation is not available yet, however.

Inhibition of the peak 2, but not peak 1, Cbl-binding activity by concanavalin A (Fig. 2) suggests that the protein includes a mannoside linkage. The result indicates that the different pI values of the two cytosolic Cbl-binding proteins are due to modification in the sugar chain.

The K_s value of the peak 1 protein for CN-Cbl was 1.0 nM and that of the peak 2 protein 2.0 nM. The Cbl-binding proteins showed lower affinity for Cbl than did TC-II and R-binder (K_s value 0.01 nM) [28], but showed a same degree of K_s values as IF (0.1–1 nM) [1,29] and periplasmic Cbl-binding protein of *E. coli* (0.3 nM) [30]. The different properties of the peak 1 and peak 2 proteins might be ascribed to the sugar chain, which is probably bound near the Cbl-binding site of the peak 2 protein. The binding of Cbl to the cytosolic Cbl-binding proteins was observed over as wide a range as pH 5.0–9.0, similarly to the cases of TC-II (pH 4–11) and IF (pH 3–9) [28]. The Cbl-binding activities of the peak 1 and peak 2 proteins were not inhibited by 2-AMP-Cbl, suggesting that the alkanolamine group of the Cbl molecule is essential for the Cbl-binding activity of *Euglena* cytosolic

binding proteins. R-binder binds many cobamides and other corrinoids with equally high affinity, whereas in the case of IF the binding affinity for Cbl is independent of the structure of the β -co-ordination position and corrin ring side chains of the Cbl molecule, although the nucleotide portion is essential for the formation of the IF–Cbl complex [31].

Diol dehydrase in *Aerobacter aerogenes* requires univalent cations for formation of the holoenzyme [32]. The IF–Cbl-complex receptor in mammals is inhibited by EDTA and re-activated by Ca^{2+} [33]. The Cbl-binding activities of the *Euglena* cytosolic Cbl-binding proteins were not affected by various metal ions. Conversion of some Cbl-dependent apoenzymes, e.g. methylmalonyl-CoA mutase in mammals, into holoenzymes is known to be inhibited by some thiol-group inhibitors [23]. In neither of the *Euglena* cytosolic Cbl-binding proteins were thiol groups involved in the Cbl-binding site, since thiol-group inhibitors did not affect their Cbl-binding activities.

Euglena ribonucleotide reductase was purified by Hamilton [17] as a Cbl-dependent enzyme with an M_r of 440 000, which is different from the M_r value of the cytosolic Cbl-binding proteins. Rosenberg and co-workers [34] have reported that the cytosolic and mitochondrial Cbl-binding proteins are identical with N^5 -methyltetrahydrofolate:homocysteine methyltransferase and methylmalonyl-CoA mutase respectively in rat liver. The occurrence of these enzymes in *Euglena* also has been suggested in our laboratory [35], but the purified cytosolic Cbl-binding proteins did not show these enzyme activities, suggesting that these Cbl-dependent enzymes assayed here are not located in cytosol or do not function as Cbl-binding proteins. That the Cbl-binding activity in cytosol was hardly inactivated by some reagents (preincubation with 0.1 % SDS, 0.1 % Triton X-100, 2 M-urea and 10 % and 20 % ethanol at 25 °C for 15 min; results not shown) gives some support to the above speculation. The results indicate that the cytosolic Cbl-binding proteins function solely for intracellular transport of Cbl and protection of Cbl from photolysis.

Daisley [24] has reported that an extracellular Cbl-binding protein appears to be derived from an intracellular binding protein in *E. gracilis*. However, it was not clear in which organelle is located the intracellular Cbl-binding protein that is linked to the extracellular Cbl-binding protein. The extracellular Cbl-binding protein that we isolated from the culture medium of *Euglena* grown for 5 days was identical with the cytosolic binding proteins, but not the mitochondrial one, as established by means of an immunodiffusion study (Fig. 3). The physiological function of the extracellular Cbl-binding protein is not clear, however.

Our previous paper [36] described the distribution of the Cbl-binding proteins in *E. gracilis* z (mitochondria, 11 %; chloroplasts, 21 %; microsomal fraction, 9 %; cytosol, 59 %). The data were the distribution of the binding capacities of the Cbl-unsaturated binders after pre-labelling of the wild-type cell with radioactive CN-Cbl for 30 min. The correct Cbl-binding activity in the wild-type cell was distributed in the mitochondria (approx. 9 %), chloroplasts (11 %), microsomal fraction (19 %) and cytosol (60 %) (calculated from the above distribution data). Although the cytosolic and microsomal contents in the wild-type cell were similar to those

in the bleached mutant, the mitochondrial ones were increased in the mutant. Our preliminary experiments have indicated that N^5 -methyltetrahydrofolate:homocysteine methyltransferase is distributed in the mitochondria, chloroplasts and cytosol in *E. gracilis* z (Y. Isegawa, Y. Nakano & S. Kitaoka, unpublished work). The increase in the mitochondrial Cbl-binding activity in the bleached mutant might suggest the induction of the mitochondrial enzyme because of disappearance of the chloroplast methionine-synthetic system.

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