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Purification and Characterization of ADP-Ribosyl Cyclase from *Euglena gracilis*

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ADP-ribosyl cyclase, which catalyzes the conversion from NAD^+ to cyclic adenosine diphosphoribose (cADPR), is proposed to participate in cell cycle regulation in *Euglena gracilis*. This enzyme, which was found as a membrane-bound protein, was purified almost the homogeneity after solubilization with deoxycholate, and found to be a monomeric protein with a molecular mass of 40 kDa. Its K_m value for NAD^+ was estimated to be 0.4 mM, and cADPR, a product of the enzyme, inhibited the enzyme competitively with respect to NAD^+ whereas another product, nicotinamide, showed noncompetitive (mixed-type) inhibition. In contrast to mammalian CD38 and BST-1, *Euglena* ADP-ribosyl cyclase lacked cADPR hydrolase activity.

Key words: ADP-ribosyl cyclase, CD38, cell cycle regulation, cyclic ADP-ribose, *Euglena gracilis*.

ADP-ribosyl cyclase, which catalyzes the synthesis of cADPR from NAD^+ with the concomitant formation of nicotinamide, has been purified from *Aplysia* ovotestis (*1*). In mammalian cells, the synthesis of cADPR from NAD^+ is catalyzed by CD38 (a T-lymphocyte marker protein) and its family (*2-6*), and by BST-1 (a glycosyl-phosphatidylinositol-anchored protein that facilitates the stroma cell-dependent growth of pre-B cell line) (*7-9*). In contrast to *Aplysia* ADP-ribosyl cyclase, CD38 and BST-1 also have the ability to hydrolyze cADPR to form ADP-ribose (*2, 3, 7*). Furthermore, NAD^+-glycohydrolase from *Streptococcus pyogenes* has been reported to possess the synthesis and hydrolysis activities of cADPR, along with the cleavage activity of the ribose-nicotinamide bond in NAD^+ (*10*).

cADPR induces Ca^{2+} release from intracellular Ca^{2+}-stores in a variety of tissues and cells (*11-15*). In sea urchin eggs, it has been shown that cADPR, like ryanodine and caffeine, activates the Ca^{2+}-induced Ca^{2+}-releasing mechanism through the ryanodine receptor, and the physiological agonist of the ryanodine receptor is proposed to be cADPR (*16*). In pancreatic β-cells, cADPR is reported to act as a regulator of insulin secretion by Ca^{2+}-dependent mechanisms (*3, 11*).

Euglena gracilis, a unicellular protist having chloroplasts, is a useful model to study cell cycle regulation, because its cell cycle is readily synchronized by application of an appropriate light-dark cycle (*17, 18*). We have found that ADP-ribosyl cyclase activity in *E. gracilis* oscillates during the cell cycle in a synchronous culture induced by a light-dark cycle, and a marked increase in the enzyme activity occurs immediately before cell division started (*19*). In addition, it has been shown that cADPR, the product of the enzyme, acts as an inducer of Ca^{2+} release from the microsomal fraction, presumably by activating the Ca^{2+}-induced Ca^{2+}-releasing mechanism through the ryanodine receptor (*20*). On the basis of these findings, it is proposed that ADP-ribosyl cyclase and its product, cADPR, participate in cell cycle regulation from the G2 phase to the M phase through Ca^{2+}-dependent mechanisms in *E. gracilis*. Recently, it has been reported that ectocellular expression of human CD38 in mammalian cells (HeLa and NIH 3T3 cells) increases intracellular Ca^{2+} and reduces doubling time (*21*). In the present paper, we report purification and some properties of *Euglena* ADP-ribosyl cyclase.

MATERIALS AND METHODS

Materials—[adenylate-^{32}P]NAD^+ (29.6 TBq/mmol) was a product of New England Nuclear. NAD^+ was obtained from Oriental Yeast (Osaka), cADPR and ADP-ribose from Seikagaku Kogyo (Tokyo), and a monoclonal antibody against human CD38 from Cosmo Bio (Tokyo).

Organism and Culture—*E. gracilis* SM-ZK, a chloroplast-lacking mutant derived from strain Z by treatment with streptomycin (*22*), was cultured heterotrophically in Koren-Hutner medium (*23*) without supplement of cyano-Cbl (vitamin B_{12}) at 25°C in the dark for 5 days. Synchronous cell division was induced by the addition of cyano-Cbl

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Abbreviations: ADP-ribose, adenosine diphosphoribose; cADPR, cyclic adenosine diphosphoribose or cyclic ADP-ribose; Cbl, cobalamin; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

into the Cbl-deficient culture at a final concentration of 10 ng/ml. Cell density was monitored using a hemocytometer.

Enzyme Purification—Six hours after the addition of cyano-Cbl into the Cbl-deficient culture, cells were harvested by centrifugation at 1,000×*g* for 5 min, washed with Buffer A (20 mM HEPES-Tris buffer, pH 7.0, containing 1 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM EGTA) supplemented with 0.34 M glucose, 12.5 mM benzamidine, 0.1 mM PMSF, 10 μM leupeptin, and 20 μg/ml soy bean trypsin inhibitor, and disrupted by sonication in the same buffer at 4°C. After removal of cell debris by centrifugation (10,000×*g*, 5 min), the supernatant obtained was used as a crude enzyme solution.

The crude enzyme solution was centrifuged at 100,000×*g* for 60 min to separate the membrane fraction, which was then suspended in Buffer A supplemented with 0.1 mM PMSF, 10 μM leupeptin, and 20 μg/ml soy bean trypsin inhibitor, and treated with 0.25% deoxycholate at 4°C for 60 min to solubilize ADP-ribosyl cyclase. After removal of insoluble debris by centrifugation (100,000×*g*, 60 min), the solubilized enzyme was applied onto a DEAE-Sepharose column (1×20 cm) that had been equilibrated with Buffer A containing 0.1 mM PMSF and 10 μM leupeptin. After washing the column with the same buffer (30 ml), ADP-ribosyl cyclase was eluted with 80 ml of a linear concentration gradient of 0-1 M NaCl in the buffer. Active fractions were combined, dialyzed against Buffer A, and put onto a DEAE-Toyopearl (1×4 cm) preequilibrated with the buffer. After washing the column with Buffer A, the enzyme was eluted by increasing the concentration of NaCl (0 to 1 M) in the buffer (30 ml). The enzyme eluted was dialyzed against Buffer A, then chromatographed on an AF-Blue-Toyopearl column (0.5×5 cm) using Buffer A (10 ml) with a linear concentration gradient of NaCl (0-1 M) as an elution buffer. After dialysis, the enzyme was further purified by Q-Sepharose column (0.5×5 cm) chromatography using Buffer A (10 ml) with an increasing concentration of NaCl (0 to 2 M). Protein content was determined according to Bradford (*24*) using bovine serum albumin as a standard.

Enzyme Assay—ADP-ribosyl cyclase was incubated with 4 mM [adenylate-^{32}P]NAD^+ (11.7 MBq/mmol) in 1 ml of 20 mM MOPS-NaOH buffer, pH 7.0, at 30°C. After an appropriate time, the reaction was stopped by addition of 0.1 ml of acetone, and cADPR produced was analyzed by a radio-HPLC system using an Inertsil ODS column (4.6×150 mm; GL Science, Tokyo) or a J'sphere ODS-M80 column (4.6×150 mm; YMC, Kyoto). The reaction mixture was placed on an ice-bath for 60 min, then centrifuged at 10,000×*g* for 10 min. The supernatant obtained was evaporated to dryness and dissolved in a small volume of water, and a portion of the solution was applied onto a column that had been equilibrated with 20 mM potassium phosphate buffer, pH 6.0, containing 2% methanol. cADPR was eluted with the buffer at a flow rate of 0.8 ml/min, and radioactivity and absorbance at 260 nm were monitored. cADPR, ADP-ribose, and NAD^+ were eluted respectively at 4, 5, and 13 min from the Inertsil ODS column, and at 4.5, 8.5, and 24 min from the J'sphere ODS-M80 column.

Determination of Molecular Mass by Gel Filtration and SDS-PAGE—To determine its molecular mass, ADP-ribosyl cyclase was chromatographed on a Superose 6 column (0.5×50 cm) using Buffer A containing 0.1 M NaCl as an elution buffer at a flow rate of 0.1 ml/min. SDS-PAGE was done on 12.5% gel according to Laemmli (*25*), and proteins in the gel were detected by silver staining.

RESUTLS AND DISCUSSION

Purification of ADP-Ribosyl Cyclase from E. gracilis—*Euglena* requires Cbl as an essential growth factor and is easily rendered Cbl-deficient (*26*), because adenosyl-Cbl acts as a coenzyme of deoxyribonucleotide reductase, which participates in DNA synthesis in this organism (*27*). *Euglena* cells were cultured heterotrophically in a Cbl-deficient medium for 5 days to arrest cell growth, then sufficient Cbl was added to induce synchronous cell division. After the addition of Cbl, DNA replication was completed in 5 h, and synchronous cell division occurred during 6 through 10 h. ADP-ribosyl cyclase activity in these cells increased remarkably (over 2-fold) just before the cell division started, then returned to its original level after cell division was complete. These observations confirm our previous finding that the ADP-ribosyl cyclase activity is temporally increased in the G2 phase of the cell cycle in *E. gracilis* (*19*).

Crude extract was prepared from *Euglena* cells in the G2 phase and centrifuged at 100,000×*g* for 60 min to separate soluble and membrane fractions. As shown in Table I, almost 90% of the ADP-ribosyl cyclase activity was recovered in the membrane fraction. This result indicates that *Euglena* ADP-ribosyl cyclase is a membrane-bound protein.

After solubilization of ADP-ribosyl cyclase from the membrane fraction of *E. gracilis* with deoxycholate, the enzyme was purified about 160-fold over the membrane fraction by DEAE-Sepharose, DEAE-Toyopearl, AF-Blue-

TABLE I. **Localization of ADP-ribosyl cyclase in *Euglena* cells.** *Euglena* cells were cultured heterotrophically in a Cbl-deficient medium for 5 days to arrest cell growth, and synchronous cell division was induced by the addition of cyano-Cbl at a final concentration of 10 ng/ml. Just before cell division started (at 6 h after the addition of Cbl), cells were harvested and disrupted by sonication to obtain crude extract. The crude extract was centrifuged at 100,000×*g* for 60 min to separate membrane and soluble fractions.

	Total activity (nmol/min)	Protein (mg)	Specific activity (nmol/mg protein/min)	Yield (%)
Crude extract	6.01	12.5	0.48	100
Membrane fraction	5.37	1.04	5.17	89
Soluble fraction	0.32	10.7	0.03	5

TABLE II. **Purification of *Euglena* ADP-ribosyl cyclase.** The membrane fraction of *Euglena* cells in the G2 phase was obtained as described in Table I, and ADP-ribosyl cyclase was purified from the membrane fraction.

	Total activity (nmol/min)	Protein (mg)	Specific activity (nmol/mg protein/min)	Yield (%)
Membrane fraction	174	34.2	5.09	100
Solubilization	165	16.0	10.3	95
DEAE-Sepharose	157	5.22	30.1	90
DEAE-Toyopearl	124	1.12	111	71
AF-Blue-Toyopearl	30.0	0.16	188	17
Q-Sepharose	24.2	0.03	807	14

Toyopearl, and Q-Sepharose column chromatographies in a yield of 14% (Table II). Analysis of the purified preparation by SDS-PAGE showed a single protein band with a molecular mass of 40 kDa (Fig. 1). Gel filtration on a Superose 6 column gave a molecular mass of 41 kDa, indicating that *Euglena* ADP-ribosyl cyclase is a monomeric protein of about 40 kDa. These characteristics resemble those of mammalian CD38 and BST-1, which are also membrane-bound/anchored proteins of about 40 kDa (*2, 7, 28*), rather than those of ADP-ribosyl cyclase from *Aplysia* ovotestis, a soluble protein of 29 kDa (*1, 29*). However, *Euglena* ADP-ribosyl cyclase was not recognized by a monoclonal antibody against human CD38 when examined by immunoblotting following SDS-PAGE. NAD^+-glycohydrolase from *Streptococcus pyogenes* is reported to be a soluble protein excreted from cells (*10*).

Catalytic Properties of Euglena ADP-Ribosyl Cyclase— ADP-ribosyl cyclase from *E. gracilis* had an optimum pH of 6.0, and about 70% of the maximum activity was found at pH 7.0. Optimum temperature for the enzyme reaction was 30°C.

Mammalian CD38 and BST-1 catalyze a hydrolase reaction from cADPR to ADP-ribose, in addition to the cyclase reaction from NAD^+ to cADPR: ADP-ribose appears along with cADPR when NAD^+ is incubated with CD38 or BST-1 (*3, 4, 6, 7*). To clarify whether *Euglena* ADP-ribosyl cyclase has a similar hydrolase activity, the purified enzyme (50 ng) was incubated with [adenylate-^{32}P]NAD^+ in 1 ml of MOPS-NaOH buffer, pH 7.0, for 30 min, and cADPR and ADP-ribose formed during the incubation were analyzed by use of a radio-HPLC system with an Inertsil ODS column. As shown in Fig. 2, the amount of labeled cADPR increased linearly during the enzyme reaction, but little labeled ADP-ribose was formed even after 30 min. The enzyme (200 ng) was also incubated with cADPR (100 μM) in the buffer (1 ml) for 120 min, and the reaction mixture was analyzed with an HPLC system using a J'sphere ODS-M80 column by monitoring absorbance at 260 nm. Again, little ADP-ribose was detected in the reaction mixture. These results indicate that ADP-ribosyl cyclase from *E. gracilis*, like the *Aplysia* enzyme (*30, 31*), does not catalyze the hydrolase reaction of cADPR, in contrast to CD38 and BST-1.

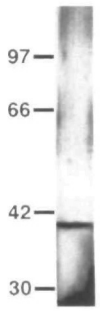

Fig. 1. **SDS-PAGE of *Euglena* ADP-ribosyl cyclase.** The purified enzyme (0.3 μg) was analyzed by SDS-PAGE (12.5% gel) and visualized by silver staining. Molecular mass (in kDa) is shown on the left.

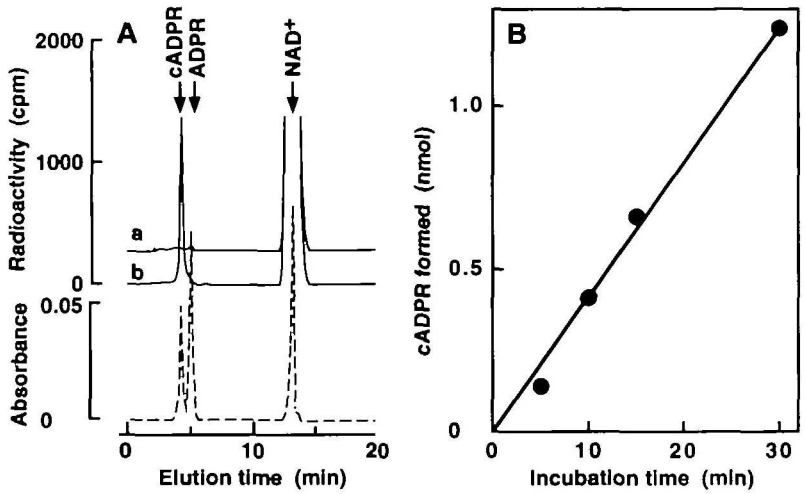

Fig. 2. **Determination of the reaction product of *Euglena* ADP-ribosyl cyclase.** Panel A: The purified enzyme (50 ng) was incubated with 4 mM [^{32}P]NAD^+ (11.7 MBq/mmol) in 1 ml of 20 mM MOPS-NaOH buffer, pH 7.0, at 30°C. After incubation for 0 (line a) or 30 (line b) min, the reaction was stopped by the addition of acetone (0.1 ml), and the supernatant obtained by centrifugation was evaporated to dryness and dissolved with water (0.1 ml). An aliquot of the solution (10 μl) was analyzed by use of a radio-HPLC system with an Inertsil ODS column. A mixture of authentic cADPR, ADP-ribose (ADPR), and NAD^+ (each 10 nmol) was applied onto the column, and absorbance at 260 nm was monitored (broken line). Panel B: The purified enzyme (50 ng) was incubated with 4 mM [^{32}P]NAD^+ in 1 ml of 20 mM MOPS-NaOH buffer, pH 7.0, at 30°C, and the formation of labeled cADPR was followed for 30 min. Data are presented as the mean of three determinations.

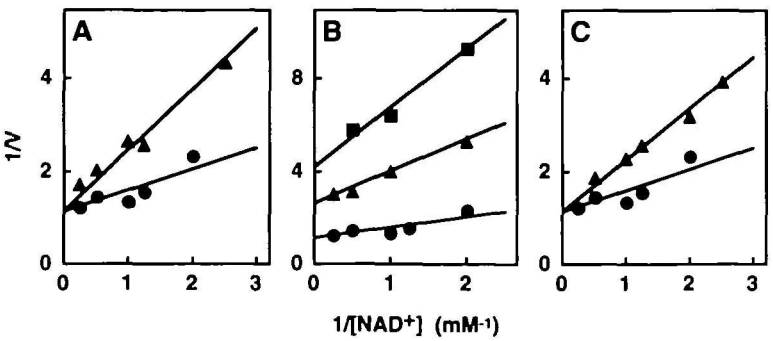

Fig. 3. **Double-reciprocal plots of the enzyme activity *versus* the NAD^+ concentration in the presence of cADPR, nicotinamide, or ADP-ribose.** The enzyme activity (V, μmol/mg protein/min) was determined with various concentrations of NAD^+ in the presence of cADPR (panel A), nicotinamide (panel B), or ADP-ribose (panel C). Panel A: cADPR was fixed at 0 (●) or 0.5 (▲) mM. Panel B: Nicotinamide was fixed at 0 (●), 0.25 (▲), or 0.5 (■) mM. Panel C: ADP-ribose was fixed at 0 (●) or 5 (▲) mM. Data are presented as the mean of three determinations.

Kinetic properties of *Euglena* ADP-ribosyl cyclase were examined to elucidate its catalytic mechanism. The enzyme reaction followed Michaelis-Menten kinetics with respect to NAD^+, and the K_m value for the substrate estimated from double-reciprocal plots was 0.4 mM (Fig. 3). cADPR, a product of the enzyme reaction, inhibited the enzyme competitively with respect to NAD^+ (Fig. 3A), whereas another product, nicotinamide, showed a noncompetitive (mixed-type) inhibition (Fig. 3B). These results reveal that the enzyme releases nicotinamide and cADPR in this order after the enzyme-substrate complex is formed. ADP-ribose, the product of the cADPR hydrolase reaction, showed competitive inhibition *versus* NAD^+, although the K_i value for ADP-ribose (about 4 mM) was over 10-fold higher than that for cADPR (about 0.3 mM) (Fig. 3C). This observation is interesting in indicating that *Euglena* ADP-ribosyl cyclase can bind ADP-ribose at its catalytic site as an analogue of cADPR, even though it shows no cADPR hydrolase activity. This is in contrast to a previous report that ADP-ribose shows no effects on *Aplysia* ADP-ribosyl cyclase (*30*). Takasawa *et al.* (*3*) reported that the hydrolase reaction of mammalian CD38 was inhibited by ATP in a competitive fashion with respect to cADPR, whereas ATP enhanced its cyclase activity; and they proposed that intracellular cADPR level was regulated by the level of ATP in mammalian cells. However, in *Euglena* ADP-ribosyl cyclase, which lacks a cADPR hydrolase activity, ATP showed no effects on its cyclase activity even at a concentration as high as 1 mM.

In summary, in this study we have purified *Euglena* ADP-ribosyl cyclase, which participates in cell cycle regulation from the G2 phase to the M phase in this organism. This enzyme, like mammalian CD38 and BST-1 (*2, 7, 28*), is a membrane-bound monomeric protein with a molecular mass of about 40 kDa, in contrast to ADP-ribosyl cyclase from *Aplysia* ovotestis, which is a soluble protein of 29 kDa (*1, 29*). However, in contrast to CD38 and BST-1, *Euglena* ADP-ribosyl cyclase resembles the *Aplysia* enzyme (*30, 31*) in lacking cADPR hydrolase activity.

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