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Euglena gracilis Ribonucleotide Reductase

THE EUKARYOTE CLASS II ENZYME AND THE POSSIBLE ANTIQUITY OF EUKARYOTE B12 DEPENDENCE[*]

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Ribonucleotide reductases provide the building blocks for DNA synthesis. Three classes of enzymes are known, differing widely in amino acid sequence but with similar structural motives and allosteric regulation. Class I occurs in eukaryotes and aerobic prokaryotes, class II occurs in aerobic and anaerobic prokaryotes, and class III occurs in anaerobic prokaryotes. The eukaryote *Euglena gracilis* contains a class II enzyme (Gleason, F. K., and Hogenkamp, H. P. (1970) *J. Biol. Chem.* 245, 4894–4899) and, thus, forms an exception. Class II enzymes depend on vitamin B_{12} for their activity. We purified the reductase from *Euglena* cells, determined partial peptide sequences, identified its cDNA, and purified the recombinant enzyme. Its amino acid sequence and general properties, including its allosteric behavior, were similar to the class II reductase from *Lactobacillus leichmannii.* Both enzymes belong to a distinct small group of reductases that unlike all other homodimeric reductases are monomeric. They compensate the loss of the second polypeptide of dimeric enzymes by a large insertion in the monomeric chain. Data base searching and sequence comparison revealed a homolog from the eukaryote *Dictyostelium discoideum* as the closest relative to the *Euglena* reductase, suggesting that the class II enzyme was present in a common, B_{12}-dependent, eukaryote ancestor.

Ribonucleotide reductases (RNRs)[3] provide all cells with the deoxyribonucleoside triphosphates (dNTPs) required for DNA replication and repair (1–4). During evolution their appearance was a prerequisite for the transition of the RNA world to the DNA world (5, 6). They catalyze the replacement of an OH group in the ribose moiety of a ribonucleoside di- or triphosphate by a hydrogen atom. Chemically this is a difficult reaction. It occurs by an unusual free radical mechanism in which a cysteine of the enzyme becomes a free radical and participates in the catalytic reaction (4, 7). The process by which the radical is generated differs between aerobic and anaerobic organisms; this has led to the definition of three different classes of ribonucleotide reductases (1–4). Class I RNRs are aerobic enzymes that are found both in eukaryotes and microorganisms. They consist of two homodimeric proteins (R1 and R2), where R2 generates a cysteine radical in R1 with the aid of a stable tyrosyl radical (8), and R1 catalyzes the reduction of the ribonucleotide (7). Class II RNRs occur only in microorganisms and function both aerobically and anaerobically. Theses enzymes consist of a single polypeptide chain whose cysteine radical is generated by adenosyl cobalamin, vitamin B_{12} (3). They are either monomers or homodimers. Class III RNRs are also exclusively present in microorganisms. They are anaerobic enzymes (9). Catalysis resides in a single protein that contains a stable oxygen-sensitive glycyl radical. The glycyl radical is formed by a second protein that is not required for catalysis once the glycyl radical has been generated (10).

The enzymes from the three classes show little sequence homology. Nevertheless, their structures and allosteric regulation are similar, making it highly likely that they evolved from a common ancestor (11). All enzymes direct their substrate specificity toward reduction of each of the four canonical ribonucleotides via an allosteric "specificity site" to which effectors are bound such that binding of ATP or dATP directs the enzyme toward reduction of cytosine or uracil nucleotides, dTTP directs the enzyme binding toward reduction of guanine nucleotides, and dGTP directs the enzyme binding toward reduction of adenine nucleotides (11). The detailed structural transitions bringing about switches in substrate specificity were recently elucidated for a class II reductase (12). Class I and III enzymes contain in addition an "activity site" where binding of ATP stimulates and dATP inhibits the overall activity of the reductase (11).

Euglena gracilis is a eukaryotic unicellular organism with a class II ribonucleotide reductase (13, 14) and represents an exception from the general rule that eukaryotes contain class I enzymes. Ribonucleoside triphosphates serve as the substrate, and enzyme activity is absolutely dependent on adenosyl cobalamin. In these respects the reductase resembles the known class II enzyme from *Lactobacillus leichmannii* and differs from mammalian class I enzymes. A highly purified form of the enzyme catalyzed the reduction of all four canonical ribonucleoside triphosphates but apparently without the strict allosteric regulation of substrate specificity found for other ribonucleotide reductases (13, 14).

The structure and function of the *Euglena* enzyme is of interest with regard to the evolution of ribonucleotide reduction, but it also bears upon the evolution of B12 dependence among eukaryotes in general. Only prokaryotes synthesize B12 (15), but numerous eukaryotes have a B12 requirement for growth. B12 dependence is widespread among animals (15) and various unicellular organisms including diatoms, chrysophytes, dinoflagellates, euglenids (16), and some chlamydomonads (17). Although isolated reports can be found for B12-dependent enzymatic reactions among fungi (18) and higher plants (19), reports of

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[3] The abbreviations used are: RNR, ribonucleotide reductase; MetH, B12-dependent methionine biosynthesis; DTT, dithiothreitol.

B12-dependent growth for either group is lacking. In humans, two B12 dependent enzymatic steps are known; they are the methylmalonyl-CoA mutase reaction (20) and the MetH reaction in methionine synthesis (15). Outside of the animals, the biochemical basis of B12 dependence is usually obscure. Very recent findings indicate that B12 dependence in the green alga *Lobomonas rostrata* and, by inference, in many other algal groups (21) involves the B12-dependent (MetH-type) methionine biosynthesis route, as in humans, via loss of the B12-independent (MetE-type) enzyme. However, supplementing B12-deficient growth media with methionine could not restore normal *Lobomonas* growth levels (21), indicating the presence of additional B12-dependent steps. B12 dependence in *Euglena* relates to its B12-dependent ribonucleotide reductase (13, 14) and methylmalonyl-CoA mutase (22), which likely form the basis of the classical *Euglena* clinical assay for diagnosing human B12 deficiency and for quantifying human serum B12 levels (23).

Here we report the purification of class II ribonucleotide reductase from *Euglena* cells grown in the presence of limiting amounts of cobalamin. From the purified product we isolated and sequenced 11 peptide fragments, which were used to obtain a corresponding *Euglena* cDNA clone. After overproduction of the relevant protein in *Escherichia coli* we found that the protein indeed was an active class II ribonucleotide reductase with properties similar, but not identical to those of the enzyme prepared from *Euglena* cells. Both its structure and allosteric properties were closely related to the *Lactobacillus* enzyme.

EXPERIMENTAL PROCEDURES

Materials—E. gracilis KLEBS, strain Z for protein purification, was donated by Prof. Paola Mariani. *E. coli* strain Rosetta(DE3)/pRARE and plasmid pET24a were from Novagen and were used for protein overexpression. Plasmid pGEM-T easy (Promega) was used for cloning PCR-amplified fragments, and *E. coli* DH5α was routinely used for cloning and propagation of plasmids. Bacteria were grown in Luria-Bertani broth. Column material for enzyme purification was from Amersham Biosciences. *E. coli* thioredoxin (24) was a gift from Prof. A. Holmgren, Stockholm.

Purification of the Enzyme from Euglena Cells—E. gracilis KLEBS, strain Z was grown into stationary phase at 26 °C in Hutner and Provasolis's liquid medium (25) on a rotary shaker under 16/8 h of light/dark photoperiods for 6 days in the laboratory of Prof. Paola Meriani in the Department of Biology, University of Padova, Italy. The amount of cobalamin in the medium was decreased to 7.5 ng/ml in place of the prescribed 1000 ng/ml (26). The final cell density (10^5 cells/ml) was only 10% of cultures grown with an excess of the vitamin, but the specific ribonucleotide reductase activity in cell extracts was 50–100 times higher. After centrifugation and washing with buffer A (30 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM DTT, 0.5 mM phenylmethanesulfonyl fluoride, 0.5 mM benzamidine, and 1 μg/ml leupeptin), the cells (7 g from 3 liters of culture) were suspended in 28 ml of buffer A and disintegrated in a French press. All further manipulations were made close to +4 °C. The suspension was centrifuged at 30,000 $\times$ *g* for 1 h, and the clear supernatant solution was precipitated with an equal volume of a neutralized saturated solution of ammonium sulfate. The precipitate obtained after centrifugation was dissolved in a small volume of buffer A and dialyzed against the same buffer until free from sulfate ions. This solution was chromatographed with a 300 ml linear gradient from 0 to 0.25 M KCl in buffer A on a 20-ml column of DE-52 equilibrated with the same buffer. Reductase activity appeared between 0.10 and 0.14 M KCl. Active fractions were combined and precipitated with solid ammonium sulfate to 80% saturation. The precipitate was collected by centrifugation and dissolved in 0.5 ml of buffer A. After clarification by centrifugation the solution was applied to a 55-ml column of Superdex-200 and eluted with buffer A. The column had been standardized with thyroglobulin (660 kDa), ferritin (443 kDa), and bovine serum albumin (66 kDa). An active symmetrical protein peak was eluted at ~500 kDa. The typical purification procedure described above gave a 37-fold purification of the enzyme with a 40% yield resulting in a final specific activity of 14 (Table 1).

TABLE 1
Purification of RNR from *Euglena* cells
Results are from a typical purification starting from 7 g of cell paste.

	mg of protein	Total units	Specific activity	Purification
Cell extract	256	97	0.38	0
Ammonium sulfate	138	70	0.51	1.3
DEAE	8.4	32	3.8	10
Superdex-200	2.2	31	14	37

Polypeptide Sequences—A fraction of the material from the Sephadex-200 column was electrophoresed on a preparative SDS-polyacrylamide gel after denaturation and alkylation of the cysteines (Fig. 1*b*). We excised the three Coomassie-stained major bands in the 80-kDa region and treated the α band and the combined $\beta+\gamma$ bands separately as described in Hellman *et al.* (27). Briefly, we incubated the destained and moistened gel pieces with LysC protease from *Achromobacter lyticus* (WAKO Chemicals GmbH, Neuss, Germany), separated the resulting peptides by reversed phase liquid chromatography on a SMART system (Amersham Biosciences), and determined the amino acid sequences of selected well separated peaks in an Applied Biosystems Model 494A sequencer, resulting in sequences of 11 well defined peptides. In some cases we also determined their molecular mass by mass spectrometry.

Identification and Cloning of Euglena Sequence—All peptide sequences from the hypothetical *Euglena* RNR matched 7 cDNA clones corresponding to a single mRNA in a collection of 10,000 *Euglena* EST sequences[4] cloned in the EcoRI site of λZapII (Stratagene). The insert of the largest of the cDNAs (pEgRNR3, 2458 nucleotides long) was excised as a pSK(+) plasmid and sequenced. It had an open reading frame for 729 amino acids. The ATG start codon was preceded by the sequence 5′-*tttttc*gtccacagtcca-3′, with the spliced leader sequence indicated in italics (data not shown). The presence of the spliced leader indicates that the cDNA is full size, and this together with peptide 80:18 suggests that the indicated methionine probably is the authentic N terminus of the protein.

Production of Recombinant Enzyme—Two sets of PCR reactions were used for the amplification of *E. gracilis* reductase gene with plasmid pEgRNR3 as a template. In the first we used primer EugRNR-2up (5′-TTAACTTT**AAGGA**GATATACATATGGCTGAAAAGGA-3′; the ribosomal binding site is in *bold*) and primer EugRNR-3lw (5′-A<u>GAGCTC</u>ATCAGAGCTTAATGCCiAGGCG-3′; the SacI site is underlined). The amplified band was used as a template for the second PCR using primer EugRNR-1up (5′-C<u>CTCTAG</u>AAATAATTTTGTT-TAACTTTAAG**AAGGA**G-3′; the XbaI site is underlined, and the ribosomal binding site is in bold) and primer EugRNR-31w. Both PCRs were carried out in 50 μl with 50 pmol of each primer and 0.2 mM concentrations of each dNTP, 5 μl of 10$\times$ PCR buffer, and 1.5 units of High Expand Taq polymerase (Roche Applied Science) at 55 °C annealing temperature. The PCR fragment was digested with XbaI and SacI, and the resulting *Euglena* fragment (2430 bp) was inserted downstream the T7 promoter of pET24a generating plasmid pETS140. This plasmid was transformed into Rosetta(DE3)/pRARE *E. coli*.

[4] W. Martin, unpublished data.

TABLE 2
Purification of recombinant *Euglena* RNR
The results are from a typical purification starting with 12 g of cell paste.

	mg of protein	Total units	Specific activity	Purification
Cell extract	456	821	1.8	0
Phenyl-Sepharose	87	620	7.1	3.9
Q-Sepharose	13	431	32	7.9
Superdex-75	2.9	209	72	40

The plasmid-containing bacteria were grown at 25 °C with 60 μg/ml kanamycin and 17 μg/ml chloramphenicol to a final density of A_{550} of 0.5, induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside for 3 h, collected by centrifugation (17.9 g of cell paste), and disrupted in an X-press in buffer B (50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 20 mM DTT, and protein inhibitors (Roche Applied Science)). After high speed centrifugation, the protein concentration was adjusted to 10 mg/ml, and the supernatant solution was first precipitated with streptomycin sulfate (final 1%) and, after a second centrifugation, with solid ammonium sulfate to 45% saturation. The collected precipitate was dissolved in a small volume of buffer B and chromatographed on a HiLoad 16/10 Phenyl-Sepharose High Performance (Amersham Biosciences) column with a 0.75 to 0 M gradient of ammonium sulfate in buffer B. Fractions were analyzed by SDS gel electrophoresis, and those with a band containing *Euglena* RNR were chromatographed on a HighLoad 16/10 Q-Sepharose High Performance column with a 0–400 mM KCl gradient in buffer B. Final purification of the enzyme was by chromatography on a Superdex-75 10/300 column with buffer B containing 200 mM KCl. Our procedure gave a 40-fold purified homogeneous protein (Fig. 1*C*) with a 25% yield (Table 2).

Assay of Reductase Activity—In standard assays the protein was incubated in 50 mM Tris-HCl, pH 8.0, 0.4 mM dATP, 60 mM DTT, 6 μM adenosylcobalamin, and 0. 7 mM [^{3}H]CTP (12996 cpm/min·pmol^{-1}) for 20 min at 25 °C in a final volume of 0.05 ml. The reaction was terminated with 0.5 ml of 1 M perchloric acid, and the amount of dCTP formed was determined by chromatography on Dowex-50 (28). The reduction of ATP and GTP was assayed under identical conditions with ^{14}C-labeled substrates and various effectors as shown in Fig. 4. In this case we terminated the reaction by immersion in a boiling water bath, transformed nucleotides to nucleosides with alkaline phosphatase, and determined the amount of labeled deoxynucleosides as described earlier (29). One enzyme unit corresponds to 1 nmol of dCTP formed per min. Specific enzyme activity is units/mg of protein. Protein was measured by the Bradford assay (30) with bovine serum albumin as standard.

Phylogenetic Analyses—RNR homologues were identified by a BLAST search of the non-redundant data base at GenBank™ (www.ncbi.nlm.nih.gov), retrieved, and aligned using ClustalW (31). Gapped positions in the alignment were removed. Protein LogDet distances (32) between protein coding sequences were determined with LDDist (33). Neighbor-Net planar graphs of splits among protein LogDet distances were constructed with Nnet (34) and visualized with Splitstree (35).

RESULTS

Isolation and Properties of RNR Purified from Euglena Cells—Our starting material was an extract of *Euglena* grown under B_{12} limitation (26) with ~100-fold higher RNR activity than extracts from cells grown in the presence of an excess of the vitamin. After a three-step purification (see "Experimental Procedures") we obtained a 37-fold purified protein with a specific activity of 14. This is very low, as in a similar assay other pure class II enzymes had specific activities ranging from 250 to 1800 (29). Gel patterns from denaturing gel electrophoresis (Fig. 1*A*) revealed an accumulation during purification of two bands between the 77- and 97-kDa markers, suggesting that one or both of these bands were derived from the reductase. After purification without protease inhibitors, the relative amount of the slowest moving band decreased, and less enzyme activity was recovered, suggesting that proteolytic degradation was a major complication. This was strongly supported from the presence of several identical peptides in both major bands (Table 3). During the last step (gel filtration on Superdex-200) a single active protein peak with an estimated molecular mass of 500 kDa eluted between the thyroglobulin and ferritin markers.

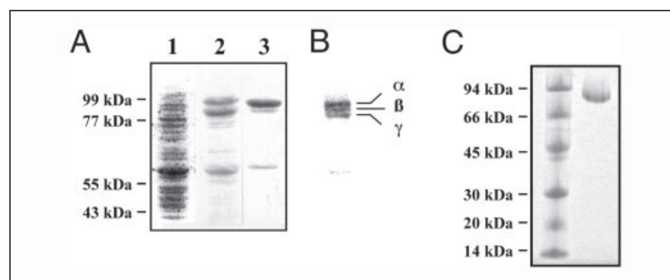

FIGURE 1. **SDS-PAGE electrophoresis of RNR from *Euglena*.** *A*, from *left* to *right*: 1) crude extract from *Euglena* cells, 2) final product from *Euglena* cells purified without protease inhibitors, and 3) with protein inhibitors. B, electrophoretic pattern of reduced and carboxymethylated RNR prepared from *Euglena* cells and used for isolation of peptides. *C*, final purified product of recombinant enzyme. In *A* and *B* the samples (4 μg) were electrophoresed on 9% polyacrylamide gels; in *C* they were electrophoresed on a 10–15% gradient gel.

Properties of the Enzyme Reaction—The purified enzyme reduced CTP (K_m = 30 μM) but not CDP. Enzyme activity absolutely required adenosyl cobalamin (K_m = 4 μM) and DTT (K_m = 20 mM). In contrast to an earlier report (14), Mg^{2+} was not required but was inhibitory at concentrations above 5 mM. Under standard conditions reduced DTT served as substitute for the physiological hydrogen donor, which probably was thioredoxin (24). Because *Euglena* thioredoxin was not available, we tested the effect of reduced *E. coli* thioredoxin and found that it was strongly stimulatory (Fig. 2). In these experiments thioredoxin was maintained in the reduced state by increasing concentrations of DTT. At low concentrations the dithiol by itself only poorly supported the reduction of CTP. The addition of thioredoxin stimulated up to 10-fold, demonstrating that *E. coli* thioredoxin was a hydrogen donor for the *Euglena* reductase. The reaction showed a broad pH optimum between 7 and 9 in Tris and phosphate buffers (data not shown). In experiments not shown here we investigated allosteric effects and found that at low concentrations of CTP the reaction was stimulated by dATP or dGTP and inhibited by dTTP. These effects largely disappeared, however, at higher concentrations of the substrate. More extensive experiments, including additional substrates and effectors, will be described in detail below for the recombinant enzyme.

Sequences of Euglena Peptides—Despite the inhomogeneity and low activity of our enzyme preparation, its behavior on gel electrophoresis suggested that it largely consisted of *Euglena* RNR. We used the preparation to determine internal peptide sequences for cloning of the enzyme as described under "Experimental Procedures" and obtained the 11 sequences given in Table 3. Because the sequenced peptides were derived by proteolysis with the highly specific LysC protease, we have added a Lys residue at the N terminus of each peptide. We obtained the sequences of peptides 81:25 and 81:46 from the β + γ band, the sequences indicated by an *asterisk* from both the α and the β + γ bands, and the remaining sequences from the α band. Sequences marked "LL" in the Table show a high degree of identity with the sequence of *L. leichmannii* RNR (36).

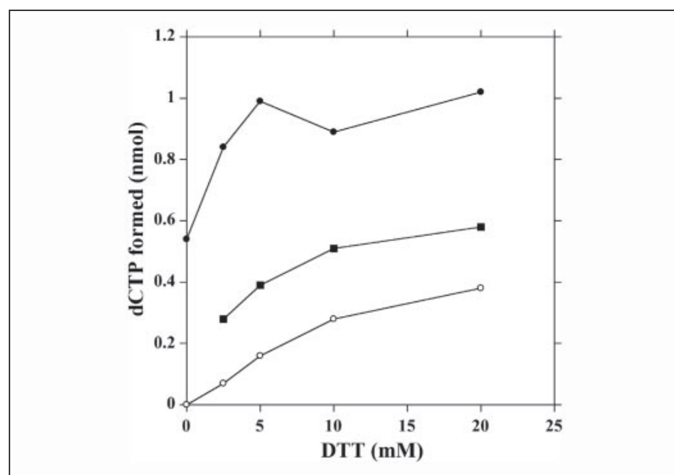

FIGURE 2. **Stimulation of ribonucleotide reduction by *E. coli* thioredoxin at increasing concentrations of DTT.** All incubations were with 25 μg of RNR purified from *Euglena* cells under standard conditions except for the concentration of DTT indicated on the *abscissa* and the addition of *E. coli* thioredoxin at 2.2 μM (■) or 22 μM (●). ○, = no thioredoxin.

TABLE 3

Peptide sequences from RNR prepared from *Euglena* cells

Peptide	Source	Sequence
81:25	$\beta + \gamma$	KFMIPDK
81:46	$\beta + \gamma$	KGISFLPRYPMGAFK
80:6	α	KI?LVGEK[LL]
80.8	α	KE?QYEEAIRK[LL]
81:39	α	KRTLYSALR?YAFEM
80:9[a]	$\alpha/\beta + \gamma$	KLHCHWDAQK[LL]
80:18[a]	$\alpha/\beta + \gamma$	KENVHPQVISSFPTPSK
80:19[a]	$\alpha/\beta + \gamma$	KREGWVESLK
80:22[a]	$\alpha/\beta + \gamma$	KTFGGTSSGPGPLINLHK[LL]
81:36	α	KEFLDLK[LL]
81:47	α	KKPPFGFNGLGEVVY[LL]

[a] Peptides present in both α and $\beta + \gamma$ bands.
[b] LL, peptides with a high degree of identity to *L. leichmannii* RNR (36).

Cloning of E. gracilis RNR and Isolation of Recombinant Enzyme—Preliminary attempts to use the peptide sequence information to clone the complete *E. gracilis* cDNA were unsuccessful. However, we found that all sequences matched the sequence from a 2458-nucleotide-long *E. gracilis* cDNA clone (pEgRNR3) that contained an open reading frame for 729 amino acids with the sequence shown in Fig. 3. The 11-peptide sequences of Table 3 are *underlined* in the figure. Data base searching revealed 13 class II sequences with >25% amino acid sequence identity to the *Euglena* enzyme across 600 or more contiguous amino acids, with the closest match observed from the eukaryote *Dictyostelium discoideum* (51% identity) followed by several *Lactobacillus* species including *L. leichmannii* (31% identity). These sequences are included for comparison in Fig. 3. Conserved residues included in all cases the catalytically important cysteines *highlighted* in Fig. 3. Interestingly, most of the residues that are functionally important in the structure of *L. leichmannii* (37) are also present in the Euglena protein (data not shown). The close relationship between the *Euglena* and *Lactobacillus* sequences suggests that the former belongs to the group of monomeric class II reductases (see "Discussion").

We overproduced the *Euglena* reductase in the *E. coli* Rosetta (DE3) strain carrying plasmid pRARE encoding all the rare codons of *E. coli* and plasmid pETS140 carrying the cDNA sequence of *Euglena* RNR. After induction with isopropyl 1-thio-β-D-galactopyranoside we obtained soluble RNR protein in bacterial extracts and purified the enzyme to homogeneity (Table 2 and Fig. 1*c*) as described under "Experimental Procedures." The protein showed an apparent molecular mass of 82 kDa on a denaturing polyacrylamide gel (Fig. 1*C*). On chromatography on Superdex-200 the homogenous protein peak eluted with a calculated molecular mass of 470 kDa. This value did not change in the presence of the allosteric effector dATP or the cofactor adenosyl cobalamin in the buffer.

Activity of the Recombinant RNR—The reaction catalyzed by the recombinant enzyme had similar properties as those catalyzed by the enzyme purified from *Euglena* cells. CTP (K_m = 0.23 mM) but not CDP served as substrate, and the reaction was absolutely dependent on DTT (K_m = 10 mM) and adenosyl cobalamin (K_m = < 1 μM). The specific enzyme activity was 72 units/mg of protein, considerably higher than that of the protein isolated from *Euglena* cells, suggesting that it was a more homogenous form of the enzyme.

With this preparation we determined the influence of nucleoside triphosphates as effectors for the reduction of the three substrates CTP, ATP, and GTP (Fig. 4). In contrast to the results with the preparation from *Euglena*, the recombinant enzyme strongly depended on appropriate effectors for the reduction of all substrates, also at moderately high substrate concentrations. The most potent effector for CTP reduction, giving the lowest K_m and highest V_{max}, was dATP (K_m = 3 μM) followed by dGTP (K_m = 18 μM) and ATP (K_m = 180 μM), with the latter two giving approximately half the V_{max} for the dATP-stimulated reaction (Fig. 4*A*). Note that high concentrations of dATP were not inhibitory, showing that the *Euglena* reductase lacks a functional activity site. Reduction of ATP (Fig. 4*B*) specifically depended on dGTP (K_m = 9 μM), and reduction of GTP (Fig. 4*c*) required dTTP (K_m = 1.3 μM) or dCTP (K_m = 3.1 μM). dTTP was a non-competitive inhibitor of the dATP-stimulated activity (Fig. 4*D*). Table 4 summarizes the data for the allosteric regulation and shows their close similarity to corresponding earlier results with the *L. leichmannii* reductase. The only clear difference is that with the latter enzyme only dTTP, and not dCTP, stimulated the reduction of GTP.

DISCUSSION

Early experiments (13, 14) had demonstrated that a RNR isolated from *Euglena* depended for its activity on adenosyl cobalamin and, therefore, functionally is a class II enzyme. There is yet no genome sequence for *Euglena*, so we cannot exclude the presence of an additional class I RNR in this protist. However, under all conditions that anyone has ever reported, the growth of *Euglena* is cobalamin-dependent. Furthermore, when the vitamin is limiting, the cells respond by overproduction of the class II RNR rather than by induced expression of a B_{12}-independent enzyme. It is, therefore, very likely that the class II enzyme represents the sole active RNR in *Euglena* under standard laboratory growing conditions.

We obtained from *Euglena* cells grown under B_{12} limitation the active RNR in highly purified but partially degraded form, sequenced the peptide fragments to clone the corresponding cDNA, overproduced the recombinant protein in *E. coli*, purified it to homogeneity, and investigated its properties. The recombinant *Euglena* enzyme differed in some aspects from the enzyme purified from the protist. The latter had a much lower specific enzyme activity and less clear-cut allosteric properties. A previously described enzyme preparation from cells grown with a full supplement of cobalamin was also deficient in allosteric regulation (13, 14). We cannot completely exclude that the differences depended on some post-translational modification absent from the recombinant enzyme. However, it is more likely that the enzyme from *Euglena* cells was partially degraded by proteolysis, resulting in both low specific activity and compromised allosteric regulation despite the presence of protease inhibitors during its preparation. Its heterogeneity on

FIGURE 3. **Sequence alignment of the *E. gracilis* B12-dependent RNR with some other monomeric class II RNR.** Comparison of the predicted amino acid sequence of the *E. gracilis* RNR (*E.gra*; AJ620503) with B12-dependent ribonucleotide reductases from *L. leichmannii* (*L.lei*; L20047), and *D. discoideum* (*D.dis*; XP_629553). *Gray shades* denote identical amino acids, and the five-cysteines involved in catalysis are *black-shaded*. Peptides described in Table 3 are *underlined* in the *Euglena* protein sequence.

denaturing gels combined with the presence of peptide sequences of the *Euglena* reductase in several electrophoretic bands are indicative of limited proteolysis. We, therefore, suggest that the results with the recombinant enzyme (Fig. 4) indeed mirror the allosteric regulation of the cellular RNR *in situ*, demonstrating that the regulation of the *Euglena* enzyme is identical to that of the *Lactobacillus* RNR (Table 4).

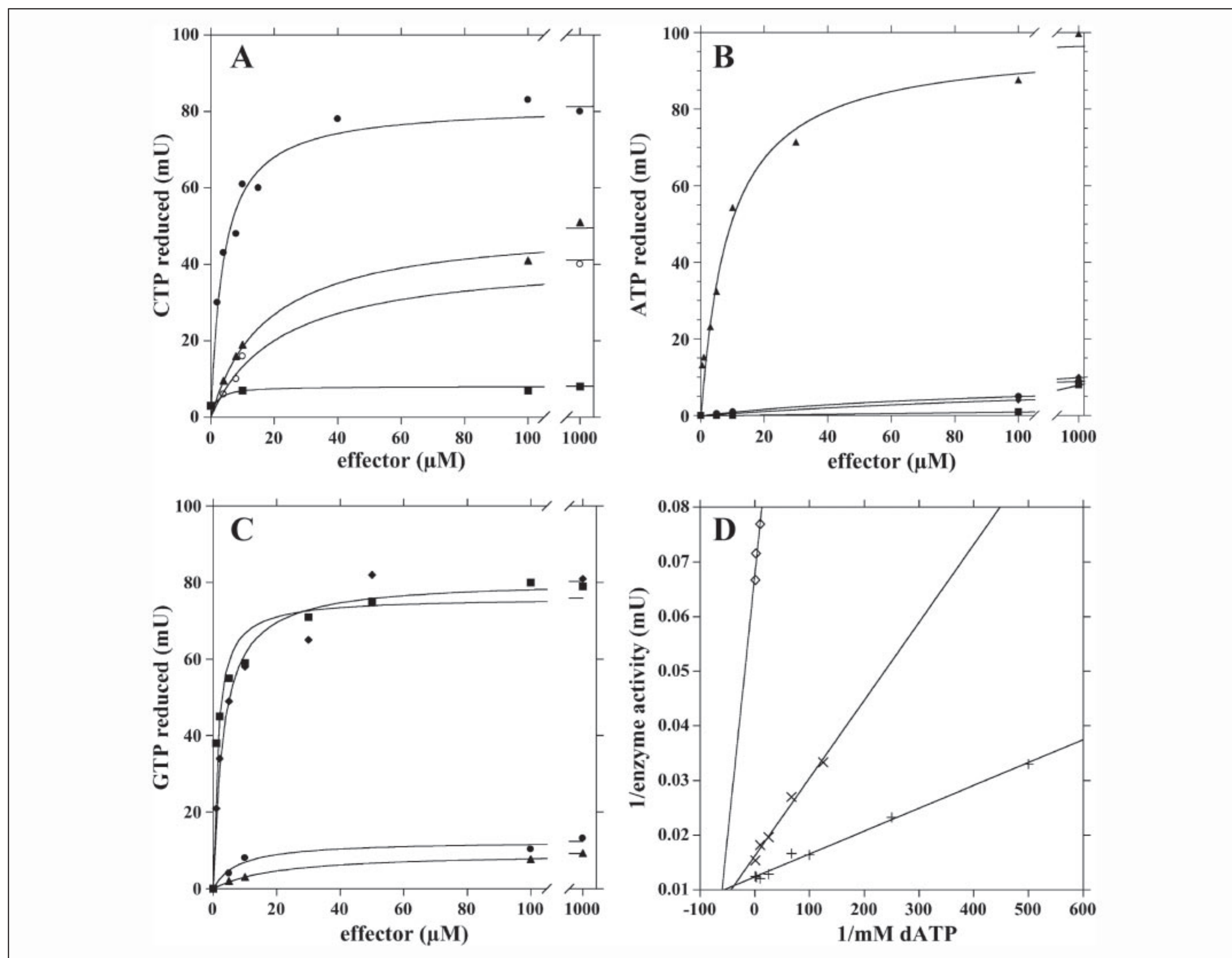

FIGURE 4. **Allosteric properties of recombinant RNR.** We measured the effects of increasing concentrations of various nucleoside triphosphates (○, ATP; ●, dATP; ▲, dGTP; ■, dTTP; ◆, dCTP) as allosteric effectors on the reduction of CTP (*panel A*), ATP (*panel B*), or GTP (*panel C*). *Panel D* shows a Lineweaver-Burk plot of the inhibition of the dATP-stimulated reduction of CTP by two concentrations of dTTP (×, 10 μM; ◇, 1000 μM; +, no dTTP). All incubations were for 20 min under standard conditions with 0.2 mM substrate and 4 μg of recombinant RNR.

TABLE 4

Comparison of the allosteric regulation of *E. gracilis* and *L. leichmannii* RNR

The *Euglena* experiments were done at 100 μM substrate concentration. Eg, *E. gracilis*; Ll, *L. leichmannii.*

Substrate	Effector											
	None		ATP		dATP		dCTP		dGTP		dTTP	
	Eg	Ll	Eg	Ll	Eg	Ll	Eg	Ll	Eg	Ll	Eg	Ll
	% relative enzyme activity											
ATP	10	10	ND[a]	ND[a]	20	10	20	10	100	100	15	15
CTP	5	10	50	90	100	100	ND[a]	ND[a]	50	15	10	20
GTP	1	15	ND[a]	ND[a]	30	60	100	25	25	ND[a]	100	100

[a] ND, not done.

Two groups of prokaryotic class II RNRs are known. Most of the currently known examples are homodimeric proteins with the *Thermotoga maritima* RNR as the prototype. Members of the second, smaller group are monomers, with the *Lactobacillus* enzyme as the prototype. Structures of both prototypes are known and show the typical fold of the catalytic site common for all RNRs, with a core 10-stranded α/β barrel. However, the allosteric specificity site differs between the two groups. The *T. maritima* enzyme binds effectors at the interphase between the two identical monomers (12), whereas the polypeptide chain of the monomeric *L. leichmannii* RNR contains a 130-amino acid insertion that mimics the part of the second polypeptide in dimeric RNRs and provides amino acids for effector binding (37). A model of the complete *Euglena* sequence closely overlaps the determined structure of the *Lactobacillus* enzyme including the insertion for the allosteric binding site,[5] strongly suggesting that the *Euglena* RNR belongs to the group of monomeric RNRs despite its multimeric behavior during gel filtration. Most likely the latter result was caused by unspecific aggregation *in vitro*. Similar to class III RNRs, the monomeric class II enzymes use triphosphates as substrates, whereas similar to class I enzymes, the dimeric enzymes use diphosphates. Also in this respect, the *Euglena* RNR falls into the group of monomeric enzymes.

Data base searching revealed that a class II RNR from *Dictyostelium* was the closest homolog to the *Euglena* enzyme (Fig. 5). The possible activity and properties of this enzyme, which coexists with a gene for a class I RNR in that genome, remain to be determined. Other close relatives besides *L. leichmannii* were RNRs from cyanobacteria and some

[5] K.-M. Larsson, unpublished information.

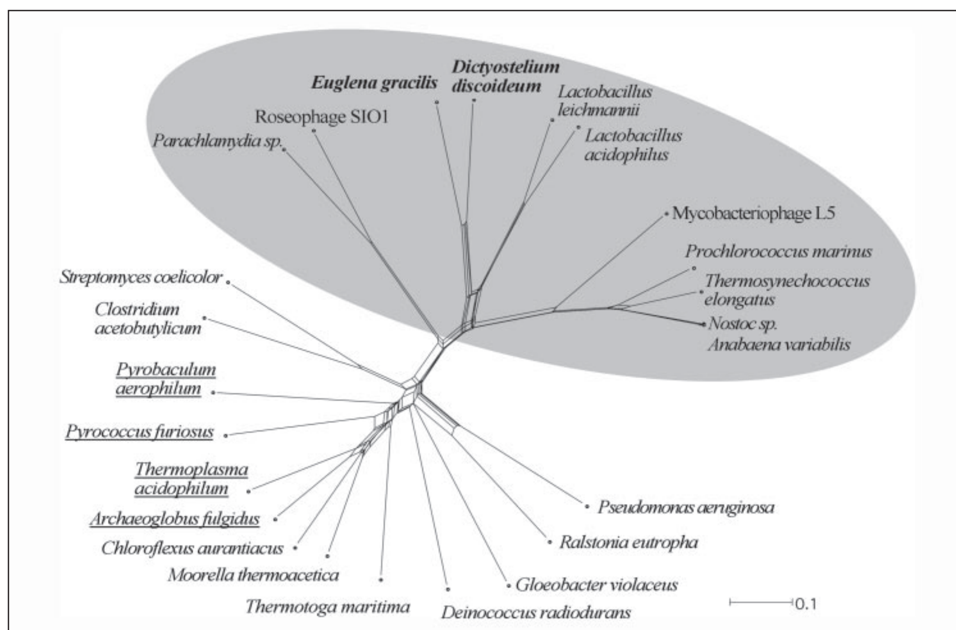

FIGURE 5. **NeighborNet planar graph of RNR sequence similarities.** The eukaryotic sequences are indicated in *bold*, and archaebacterial sequences are *underlined*. Monomeric enzyme sequences are marked with a *gray background*. Protein sequences were obtained from the ribonucleotide reductase data base (rnrdb.molbio.su.se).

phages. A homogeneous cyanobacterial RNR was shown to be monomeric (38), and the high sequence homology in the group strongly suggests that all enzymes are monomers. Indeed, the sequence identity between *Euglena* RNR and other monomeric RNRs with the homodimeric *T. maritima* RNR is very low (<15%), demonstrating the existence of distinct clusters of monomeric and dimeric RNRs (Fig. 5). Of particular interest for the present discussion is the very close relation between the two eukaryotic enzymes from *Euglena* and *Dictyostelium*.

The evolution of ribonucleotide reduction has two facets. The first is the relationship of the three different classes to one another, which is connected to the appearance of oxygen on earth. Class III enzymes are strict anaerobes and appeared first during evolution (5, 6), class II tolerates but does not require oxygen and likely evolved from class III, whereas class I depends on oxygen and evolved from class II (Fig. 6). All class I RNRs are homodimeric proteins, and the structure of their allosteric specificity site (1–4) closely resembles that of *T. maritima* (12). Class I RNRs, therefore, most likely evolved from a homodimeric class II enzyme. We propose that the monomeric class II RNRs arose by the loss of one of the polypeptides of the dimeric form and compensated this loss by the 130-amino acid insertion (Fig. 6).

The second facet concerns the distribution of class I, II, and III genes across genomes. A comprehensive survey of RNR gene presence (rnrdb.molbio.su.se/protein_index.html) reveals no clear correlation between RNR gene distribution and phylogeny, suggesting that RNR gene exchange among prokaryotes has occurred to some extent during evolution. Any combination of RNR types I, II, III, I + II, I +III, II + III or even I+ II + III can be found among prokaryotes. The physiological reasons for this are mostly obscure. In eukaryotes only class I is present with the exception of *Euglena* and *Dictyostelium*.

Why do these two primitive organisms contain a gene for a B_{12}-dependent class II RNR different from other eukaryotes? There are two simple possibilities, lateral gene transfer or inheritance from the eukaryote common ancestor. Although it is possible that both *Euglena* and *Dictyostelium* acquired their RNR genes via lateral gene transfer, the close sequence relationship between the two eukaryote genes would indicate that they were obtained independently from the same prokaryotic source, which is possible but not the interpretation that we would favor. A possible plastid (cyanobacterial) origin of *Euglena* would fail to account for the *Dictyostelium* gene.

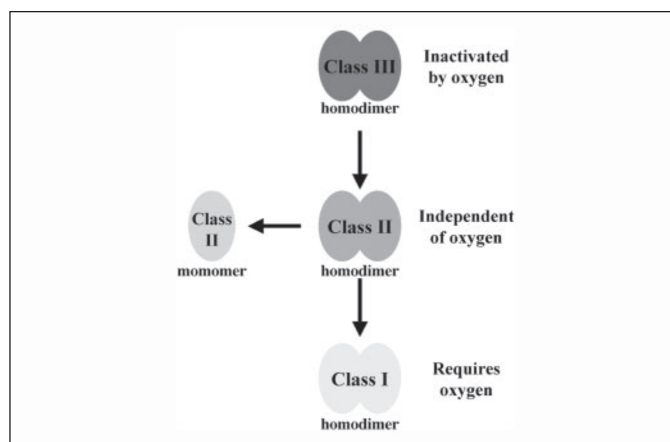

FIGURE 6. **Hypothetical scheme for the interrelations between different classes of RNRs during evolution.**

Common ancestry might be a preferable interpretation. New evidence indicates the root of the eukaryote tree to lie between the *Dictyostelium* (amoebozoa) and *Euglena* (kinetoplastid) lineages (39). Hence, the presence of a clearly homologous RNR gene in the two lineages could suggest that both a class I and II RNR was present in the common ancestor of all eukaryotes (vertical descent) followed by differential loss in several independent lineages. *Euglena* sustains prolonged anaerobiosis with the help of a highly adapted wax ester fermentation (40, 41). Although the slime mold *Dictyostelium* is not a strict anaerobe, its relatives *Entamoeba* and *Mastigamoeba* are (42). The presence of an oxygen-independent class II RNR facilitates anaerobiosis.

The eukaryote common ancestor may, thus, have been B_{12}-dependent for ribonucleotide reduction, at least under some growth conditions. The prevalence of B_{12} dependence among diverse eukaryotic lineages (15, 16) would be compatible with that view. Moreover, the newly uncovered methionine-related B_{12} dependence that is shared between some algae and animals (21) would constitute an additional B_{12}-dependent step in the eukaryote common ancestor (at least under some growth

conditions) because the plant and animal lineages also lie on opposite sides of the presently accepted eukaryotic root (39). Similar reasoning applies to B_{12}-dependent methylmalonyl-CoA mutase in animals and *Euglena* (22). From this it would not, however, follow that the eukaryote ancestor was capable of B_{12} synthesis, because many B_{12}-dependent microbes are B_{12} auxotrophs (15), which obtain the vitamin from the environment. Conversely, had the ancestral eukaryote been B_{12}-independent, then at least three B_{12}-dependent biochemical conversions (RNR, methylmalonyl-CoA mutase and MetH) would have arisen or would have been acquired in distinct eukaryote lineages independently, which seems unlikely.

Thus, although the origin of eukaryote class II RNR cannot be firmly resolved with the present sparse sample of closely related homologues, the available data would favor the interpretation that the class II RNR gene was present in the eukaryote common ancestor, which was, by inference, probably vitamin B_{12}-dependent.

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