**Euglena gracilis** ascorbate peroxidase forms an intramolecular dimeric structure: its unique molecular characterization

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**INTRODUCTION**

APX (ascorbate peroxidase, EC 1.11.1.11) is widely distributed in plants, eukaryotic algae and protozoa that have acquired the ability to synthesize AsA (ascorbate) and catalyses the reduction of H$_2$O$_2$ to water with AsA as its specific electron donor [1–3]. APX plays a central role not only in the scavenging of excess H$_2$O$_2$, to protect cells from oxidative damage, but also in the regulation of cellular redox status, in response to environmental or physiological conditions, to evoke a redox signal transduction [1–4].

Genome-wide analyses have indicated that plant APXs belong to a multigenic family, as there are nine genes in Arabidopsis, eight in rice and seven in tomato [5–7]. The APX isoenzymes are further classified into three subfamilies according to subcellular localization: those in the cytosol, microbodies, stroma and further classified into three subfamilies according to subcellular localization: those in the cytosol, microbodies, stroma and thylakoid-membrane-bound forms, have a very similar structure and thylakoid-membrane-bound forms, have a very similar structure. These findings concerning the number and subcellular localization of APX in eukaryotic algae suggest a cellular metabolism of H$_2$O$_2$ different from that in higher plants. Moreover, a few studies have examined the molecular characterization and physiological importance of algal APXs. We previously purified and characterized Euglena APX [13]. The native enzyme was monomeric with a molecular mass of 58 kDa, nearly twice as large as cytosolic APX proteins in higher plants. Another interesting aspect of Euglena APX was that, apart from H$_2$O$_2$, the enzyme can also reduce alkyl hydroperoxides, suggesting that like the glutathione peroxidase of animals, the enzyme can protect from oxidative damage. However, the substrate specificity of the native enzyme was different from that of APX-N and APX-C. The APX-N and APX-C domains are quite limited in number and distribution. The APX of Euglena gracilis is restricted to the cytosol with no isoform found in chloroplasts or other organelles [8]. Furthermore, the AsA-glutathione cycle involving monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase was exclusively found to reside in the cytosol [9]. Similarly, the APXs of Chlorella and Galdieria are distributed only in the cytosol [10,11], whereas the Chlamydomonas APX occurs only in the stroma of chloroplasts [12]. These findings concerning the number and subcellular localization of APX in eukaryotic algae suggest a cellular metabolism of H$_2$O$_2$ different from that in higher plants. Moreover, a few studies have examined the molecular characterization and physiological importance of algal APXs. We previously purified and characterized Euglena APX [13]. The native enzyme was monomeric with a molecular mass of 58 kDa, nearly twice as large as cytosolic APX proteins in higher plants. Another interesting aspect of Euglena APX was that, apart from H$_2$O$_2$, the enzyme can also reduce alkyl hydroperoxides, suggesting that like the glutathione peroxidase of animals, the enzyme can protect from oxidative damage.
Euglena gracilis, strain Z, was maintained by regular subculturing and was grown in Koren–Hutner medium under continuous illumination (24 μmol m⁻² s⁻¹) at 26 °C for 6 days, by which time the stationary phase was reached [14].

Cloning of a full-length cDNA encoding Euglena APX

Using the Euglena EST (expressed sequence tag) database from the Protest EST Program, gene-specific primers were designed to amplify the missing 5’ end of the APX EST (ELL0005171). The primers were EgAPX-SP1 (5′-GACACCCGTGGGTTGATTC-3′) and EgAPX-SP2 (5′-ACCCACGTGCCAGCTCC-3′) and were used sequentially for 5’-RACE (rapid amplification of cDNA ends) with a GeneRacer kit (Invitrogen). Then, based on a partial cDNA sequence identified previously [15], the full-length coding sequence of Euglena APX was amplified by PCR with EgAPX-F (5′-GAGCTGCGACGTTGGGTGCCGGCCTTG-3′) and EgAPX-R (5′-GCAGACCGGGGGGAAGGCGGCGGACCCGGTG-3′). PCR amplification was carried out using a GC-rich PCR kit (Roche Diagnostics).

Expression and purification of the recombinant APX

The cDNAs for the mature FL-APX and each truncated version (APX-N and APX-C) were amplified from the full-length cDNA as a template by PCR using as primers: EgAPX-NF, 5′-ctcggagGAGCTGCCGACGTTGGGTGC-3′; EgAPX-NR, 5′-aagttctGAGCTCCCGACCCCGACCTC-3′; EgAPX-CF, 5′-ctcggagTACGGCCGCTGGCCGACCGAGCCGGTG-3′; and EgAPX-CR, 5′-AAGctGCGCCGCATGTTGGCTGCGGC-3′ in order to introduce XhoI and HindIII sites containing stop codons into the 3′ ends of the cDNAs as the need arises (indicated by lowercase letters). The amplified fragments were cloned into a pGEM-T easy vector (Promega) to confirm the absence of PCR errors. The amplified fragments were cloned into a pGEM-T easy vector (Promega) to confirm the absence of PCR errors. The amplified fragments were cloned into a pGEM-T easy vector (Promega) to confirm the absence of PCR errors.

The recombinant APX-N and APX-C enzymes were mixed together to an Immobilon PVDF membrane (Millipore) and blotted using the Coomassie Blue protein assay reagent (Bio-Rad Laboratories).

Gel-filtration analysis

The recombinant APX-N and APX-C enzymes were mixed and incubated with an equal amount of protein (0.4 mg) at 4 °C for 1 h. The samples were then subjected to chromatography on a Superdex 200 column (1.0 cm × 30 cm; GE Healthcare) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM AsA and 150 mM NaCl. The column was calibrated with Blue Dextran 2000, albumin, ovalbumin, chymotrypsinogen A and ribonuclease A standards (GE Healthcare). Fractions (0.5 ml) were collected, and the positions of the APX elution were determined by a direct assay of the enzyme activity.

Immunoblot analysis

Proteins were separated by SDS/PAGE (12.5 % gel) and blotted onto an Immobilon PVDF membrane (Millipore), with transfer buffer described previously [16] using a semi-dry electrophoretic apparatus (Taitec). The blot was incubated with a monoclonal antibody (EAP1; [13]) raised against the purified Euglena APX, which was then detected using a secondary horseradish peroxidase-conjugated goat anti-(mouse IgG) antibody (Cappel) and Western Lighting Chemiluminescence Reagent Plus (PerkinElmer) using a luminescence imager (AE-6962C, ATTO).

Genomic Southern blotting analysis

Genomic DNA was isolated from Euglena using a standard method, as described previously [17]. The DNA (20 μg) was digested overnight with HindIII or SacI and separated on a 0.8 % agarose gel. The gel was blotted onto a Hybond N + membrane (Amersham Pharmacia Biotech) and hybridized with [α-32P]dCTP-labelled probes. The probes were prepared.
PCR with genomic DNA

A partial *Euglena* APX gene inserted between the 3′-region of APX-N and 5′-region of APX-C was amplified from the genomic DNA by PCR using primers EgAPX-GF (5′-GTTTTCGGGACTTGC-3′) and EgAPX-GR (5′-GCGACGCCCTTCGGCCAG-3′). The PCR product was cloned into the pGEM-T easy vector and the resulting plasmid was sequenced using a capillary DNA sequencer (ABI PRISM 3100-Avant; Applied Biosystems).

Cell fractionation

A cell homogenate was obtained by a partial trypsin digestion of the pellet followed by mild mechanical disruption, and subcellular fractionation by differential centrifugation was performed as described previously [9].

Estimation of chlorophyll content

Chlorophyll was extracted from *Euglena* cells with 80% (v/v) acetone and its level estimated using the formula: total chlorophyll \(=\frac{A_{663} \times 2.0394}{A_{650}}\) + chlorophyll \(=\frac{A_{663} \times 2.66 \times A_{650}}{A_{650}}\).

RNAi (RNA interference) experiments

Silencing of APX by RNAi was performed as described previously [18]. An approx. 450-bp partial *Euglena* APX cDNA was PCR-amplified with the addition of the T7 RNA polymerase promoter sequence (underlined in the primer sequences below) at one end. The primers were EgAPX/ RNAi-F (5′-TAATACGACTACTATAGGGACGGAGGATCATGGCC-3′) and EgAPX/RNAi-R (5′-TAATACGACTACTATAGGGACGGAGGATCATGGCC-3′), and a polypeptide chain EF1-α (elongation factor 1-α) from *Euglena* (accession no. X16890; EF1α-F, 5′-ACAGATTGGAACGGTACG-3′; EF1α-R, 5′-CAGATTTCTCTACACCATG-3′) as a normalizer.

Detection of cellular H2O2

The intercellular production of H2O2 was measured using an H2O2-specific BES (benzenesulfonyl) derivative of a fluorescein probe, BES-H2O2-Ac (Wako). BES-H2O2-Ac was added to the cells at a final concentration of 10 μM. After a 30-min incubation at room temperature, the cells were collected by microcentrifugation (1000 g for 5 min) and the supernatant removed. Fluorescence was observed under a fluorescence microscope (BX51; Olympus) with excitation and emission wavelengths set at 485 and 530 nm respectively.

RESULTS

*Euglena* APX contains two homologous catalytic domains, forming an intramolecular dimeric structure

To isolate a full-length cDNA encoding *Euglena* APX, we adopted a PCR-based oligo-capped method utilizing primers corresponding to sequences of an EST clone and a partial cDNA isolated previously [19]. The final clone containing the full-length cDNA, as indicated by the presence of a 5′-TTTCTTCG-3′ spliced leader at the 5′ end [20], was 2403 bp long with a coding region of 1644 nucleotides and encoded a protein of 649 amino acid residues (see Supplementary Figure S1 available at http://www.BiochemJ.org/bj/426/bj4260125add.htm). The cDNA obtained had an exceptionally high GC content of 70.8%. The N-terminal and internal peptide sequences that were identified previously in the purified *Euglena* APX could be found in the deduced amino acid sequence of the cDNA [13]. The exact matching of these amino acid sequences confirmed the authenticity of the cDNA. As judged from the N-terminal sequence of the purified APX, the deduced amino acid sequence had an N-terminal extension of 102 residues. A detailed analysis of this extension will be described below. Therefore the predicted mature FL-APX consisted of 547 amino acids with a molecular mass of 60075 Da, which approximately agreed with the value obtained previously by SDS/PAGE for the purified native enzyme [13].

Interestingly, the primary structure of the *Euglena* APX reveals that it contained two homologous catalytic domains (designated APX-N and APX-C), which were 68.9% identical with each other (Figure 1). Both domains had conserved distal and proximal histidine residues and contained all of the residues known to be essential for catalysis among the APX proteins in higher plants [2]. The *Euglena* enzyme is the only APX known to have two catalytic domains. Computer-assisted comparison of the deduced amino acid sequences of APX-N and APX-C with sequences in the database revealed approx. 38–54% sequence identity with APX proteins from various species. The phylogenetic relationships of various APX proteins, including *Euglena* APX, were determined by constructing an unrooted tree using the ClustalW program (Figure 2). This suggested that the two *Euglena* APX domains are closest to that of *Trypansomos cruzi* and *Leishmania major*, separating into their own protozoan clade. In this clade, there is a common feature distinguishing the enzymes from other plant-type APX families; the presence of a 16-amino-acid insertion near the C-terminal region (Figure 1B).
To gain information on the structure of the *Euglena* APX, harbouring the two homologous domains, a computer model for the three-dimensional structure was constructed. Pea cytosolic APX (PDB code 1APX), having 54% and 49% sequence identity with the APX-N and APX-C domains respectively, was selected as the template for modelling, which was performed with the Molecular Operating Environment molecular graphics package (Chemical Computing Group). The best model was chosen using the energy function included with the software.

As shown in Figure 3, the superimposed model indicated that the overall structure of each domain matched well with that of the pea cytosolic APX, suggesting that the *Euglena* APX forms an intramolecular dimer, hinged by the middle of the structure including residues 377–385.

**Subcellular localization of APX in *Euglena***

As described above, the deduced *Euglena* APX had an N-terminal extension of 102 amino acid residues. The TMHMM (transmembrane helices based on a hidden Markov model) program [20a] for the estimation of potential membrane-spanning regions showed the presence of one significant hydrophobic region in the extension (see Supplementary Figure S2 available at http://www.BiochemJ.org/bj/426/bj4260125add.htm), a typical feature of *Euglena* class II plastid signals proposed by Durnford and Gray [21]. Therefore to re-assess the cellular localization of APX in *Euglena*, we carried out subcellular fractionation by differential centrifugation. The activity and protein of *Euglena* APX were found only in the cytosol, not in organelles including chloroplasts (Figure 4).

**Genomic Southern blot and PCR analysis of the APX gene***

We next performed genomic Southern hybridization. The genomic DNA was digested with HindIII and SacI. The former enzyme does not digest within the ORF (open reading frame) of APX and the latter recognizes the border region between APX-N and APX-C. When the cDNA fragments prepared for the mature FL-APX, APX-N and APX-C were used as the probe, several hybridization signals were detected with almost the same pattern (Figure 5). These observations suggest that the *Euglena* genome has multiple copies of the APX gene. Then, PCR of the genomic DNA, utilizing primers from the 3′- and 5′-terminal sites of the APX-N and APX-C domains respectively was performed, yielding a single band. Sequencing revealed that both the APX-N and APX-C domains were in the same segment of the gene and in a *cis*-configuration, separated by an intron containing 536 nucleotides (see Supplementary Figure S3 available at http://www.BiochemJ.org/bj/426/bj4260125add.htm).
Molecular characterization of Euglena ascorbate peroxidase

Figure 2  Phylogenic tree for Euglena APX and other orthologous APX proteins

The phylogenic tree was constructed using the ClustalW program and visualized with TreeView. For the analysis, the amino acid regions 103–376 and 377–649 were used as the template of APX-N and APX-C respectively. The abbreviations and UniProt accession numbers for the APX orthologues are as follows: At, Arabidopsis thaliana (At.APX1, X59600; At.sAPX, X98925; At.tAPX, X98926); Cka, Cucurbita cv. Kurokawa Amakuri (Cka.mAPX, AB070626; Cka.tAPX, D83658); Gm, Glycine max (Gm.APX1, L10292; Hv, Hordeum vulgare (Hv.APX1, AB003117); Mc, Mesembryanthemum crystallinum (Mc.mAPX, U43561; Mc.tAPX, AF139315); Nt, Nicotiana tabacum (Nt.APX1, D85912; Nt.tAPX, AB022273); Os, Oryza sativa (Os.APX1, D45423; Os.APX2, AB053243; Os.APX3, AY382617); So, Spinacia oleracea (So.APX1, D85864; So.APX2, D49679; So.mAPX, D84104; So.tAPX, D77997); Ps, Pisum sativum (Ps.APX, X62077); W80, Chlamydomonas sp. W80 (W80.sAPX, AB003084); Cr, Chlamydomonas reinhardtii (Cr.sAPX, AJ223325); Tc, Trypanosoma cruzi (Tc.APX, AJ455787); Lm, Leishmania major (Lm.APX, XM_001686044); and Yl, Yarrowia lipolytica (Yl.APX, XM_503271).

Figure 3  Hypothetical structure model of Euglena APX

The model was generated based on pea cytosolic APX (PDB code 1APX). Euglena APX and pea cytosolic APX are shown in blue and yellow green respectively. The haem molecules are indicated as a stick format.
Subcellular localization of APX in Euglena

Subcellular fractionation of lysate from Euglena cells was performed as described in the Experimental section. Aliquots of fractions containing an equal amount of protein (20 μg) were subjected to SDS/PAGE (12.5% gel) and analysed by immunoblotting using the EAP1 monoclonal antibody. The amount of chlorophyll and the level of APX activity in each fraction are also shown. sup, supernatant; ppt, pellet.

Enzymological properties of the recombinant Euglena APX

We prepared the recombinant mature FL-APX and the APX-N and APX-C domains, and then compared their catalytic activities. As shown in Figure 6A, the recombinant proteins expressed in E. coli were purified to homogeneity by SDS/PAGE, and had a size of 60 kDa and 30 kDa for the mature FL-APX and the individual domains respectively, in good agreement with the molecular mass calculated from the predicted amino acid sequence. The recombinant enzymes showed an absorption spectrum characteristic of the high-spin ferric state of haem proteins. Soret absorption peaks for recombinant FL-APX, APX-N and APX-C were found at 410, 413 and 411 nm respectively and shifted to 430, 431 and 435 nm after reduction of each protein by the addition of dithionate (Figure 6B). In addition, cyanide complexes of recombinant FL-APX, APX-N and APX-C exhibited peaks at 421, 421 and 424 nm respectively and additional peaks at 542, 542 and 540 nm. Table 1 shows a comparison of the enzymatic properties of recombinant and native APXs. The recombinant enzymes reduced H2O2 using AsA, obeying Michaelis–Menten kinetics, with apparent $K_m$ values for both AsA and H2O2 comparable with those of the native enzyme; however, APX-N and APX-C had $k_{cat}$ and $k_{cat}/K_m$ values approx. 2-fold lower than FL-APX and the native APX. Moreover, the substrate specificity of the mature FL-APX differed from that of the truncated enzymes. In a similar manner to the native enzyme, the recombinant FL-APX exhibited significant activity toward alkyl hydroperoxides, such as t-butyl hydroperoxide and cumene hydroperoxide, whereas neither APX-N nor APX-C had any activity. The purified APX-N and APX-C domains mixed in a 1:1 molar ratio also showed no activity toward alkyl hydroperoxides (results not shown). No dimerization of APX on the simple incubation of APX-N and APX-C was observed; a protein peak at approx. 60 kDa was not found during size-exclusion chromatography with the recombinant protein mixture (results not shown).

Effect of suppressing APX on the cellular H2O2 level

To determine the physiological role of APX in the metabolism of H2O2 in Euglena cells, we temporarily suppressed APX expression using RNAi. A dsRNA synthesized from part of the Euglena APX sequence, corresponding to the APX-C domain, was introduced into Euglena cells by electroporation. No significant difference was observed in phenotype, including cell growth and cell shape, between dsRNA-containing cells and control cells electroporated with buffer only (results not shown). RT–PCR of the dsRNA-containing cells showed only a faint amplified band for the target APX, indicating that endogenous APX mRNA is degraded after introduction of the dsRNA (Figure 7A). The APX activity of dsRNA-containing cells decreased to approx. 20% of the control value (Figure 7B).

To determine intracellular H2O2 levels in control and dsRNA-containing cells, we used a chemical fluorescent probe, BES-H2O2-Ac, recently developed for the non-invasive measurement of intercellular H2O2 in vivo [22]. As shown in Figure 7(C), dsRNA-containing cells showed a prominent increase in fluorescence of the probe, indicating an accumulation of cellular H2O2 upon the suppression of APX expression.

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Table 1  Comparison of substrate specificity and kinetic parameters of the recombinant Euglena APX with those of the native and soybean enzyme

Donor and peroxide specificities were determined using 0.1 mM H2O2 and 0.4 mM AsA respectively, and are the mean of three determinations. The kinetics values are estimated from the data of three replicate experiments and are means ± S.D. Statistical analysis by Student's t test indicated that all values in each row were significantly different at P < 0.05 except the pairs indicated by a, b, c and d. CumOOH, cumene hydroperoxide; n.d., not determined; t-BOOH, t-buty1 hydroperoxide; −, not tested.

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<th>rAPX-C</th>
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Figure 6  SDS/PAGE and absorption spectra of purified recombinant Euglena APX proteins

(A) The recombinant His6-tagged proteins were extracted from E. coli BL21 Star cells transformed with pColdI containing the mature FL-APX (rFL-APX), in which the first 102 amino acids had been removed and the truncated APX, APX-N (rAPX-N) and APX-C domains (rAPX-C) were then purified in a Ni-NTA (Ni2+-nitrilotriacetate) column. The samples were separated by SDS/PAGE and visualized with Coomassie Brilliant Blue. (B) The absorption spectrum of each APX (1 μM) was observed in 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM AsA and 1 mM EDTA. The reduced and CN−-bound forms were measured by the addition of 1 mM dithionite (+dithionate) and 1 mM KCN (+KCN) respectively.

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the metabolism of H$_2$O$_2$.

Microscopy. Cellular H$_2$O$_2$ was measured using a H$_2$O$_2$-specific fluorescence probe, BES-H$_2$O$_2$-Ac. Cultures of representative cells were photographed 1 week after the dsRNA was introduced.

Thirdly, the suppression of APX expression via the introduction of dsRNA confirmed that APX and EF1-α are conserved in the Euglena protozoan-type APX families than plant APXs (Figures 1 and 2). The sequences also contained the metal-binding five-residue K$^+$ site (comprising Asp-187, Thr-164, Thr-180, Asn-182 and Ile-185) [28], although Ile-185 was replaced by a lysine residue and a valine residue in APX-N and APX-C respectively.

A comparison of cytosolic APX from soybean with cytochrome c peroxidase showed that APX does not contain an additional loop structure which blocks the AsA-binding site of cytochrome c peroxidase (residues 34–41) [24]. The sequence alignment (Figure 1) and structure model (Figure 3) indicated that the loop structure of Euglena APX was missing in both the APX-N and APX-C domains, as well as in cytosolic APX from soybean and pea. Therefore, consistent with the idea that the truncated recombinant enzymes would be catalytically active, we found the kinetics of the recombinant APX-N and APX-C domains did not show any activity towards alkyl hydroperoxide (Table 1). On the other hand, the recombinant FL-APX exhibited catalytic activity towards alkyl hydroperoxide, like the native enzyme, indicating that the intramolecular dimeric structure is correlated with substrate recognition. Badyal et al. [29] have recently reported that cytosolic APX potentially has a catalytic role.

**DISCUSSION**

*Euglena* APX has unique biochemical properties among the APX isoenzymes reported to date [13]. The molecular mass of the enzyme is twice that of plant APXs. Moreover, the plant isoenzymes are highly specific for H$_2$O$_2$ as an electron acceptor, whereas *Euglena* APX shows a broad specificity, recognizing alkyl hydroperoxide in addition to H$_2$O$_2$ [13]. On the basis of the results in the present study, we draw three conclusions that are relevant to the function and physiological role of APX in *Euglena*. First, sequencing revealed that *Euglena* APX consists of two homologous domains (APX-N and APX-C) and hence it has a unique intramolecular dimeric structure, which is highly relevant to its ability to recognize substrates. Second, *Euglena* APX was distributed only in the cytosol after cleavage of the N-terminal extension typical of a class II plastid signal in *Euglena*. Thirdly, the suppression of APX expression via the introduction of dsRNA confirmed that *Euglena* APX plays a significant role in the metabolism of H$_2$O$_2$.

**Molecular and enzymatic properties of *Euglena* APX**

The primary structure of *Euglena* APX proved to be unique because of the presence of two nearly identical domains, APX-N and APX-C, and the 102-amino-acid extension in the N-terminal region (Figure 1). It is worth noting that the dimerized form of the mature FL-APX ‘intramolecular dimer’ is a novel structure, with tandem homologous domains in a single polypeptide. A comparison of the deduced amino acid sequence of the two domains of *Euglena* APX with those from known APX proteins revealed that the domains are similar to almost all the catalytic regions of the plant isoenzymes, and are more closely related to protozoan-type APX families than plant APXs (Figures 1 and 2). Previous crystallographic studies of pea and soybean cytosolic APX have identified several amino acid residues involved in substrate recognition, the reaction mechanism and stabilizing the enzyme [23–25]. Curiously, most of those key residues are conserved in the *Euglena* APX. APX is classified as a haem-containing Class I peroxidase [26]. This class shares a common feature, the presence of distal and proximal histidine-containing regions for binding a haem ligand. From sequence alignments, as shown in Figure 1, the conserved distal and proximal histidine residues of APX-N and APX-C are His-159 and His-417, and His-283 and His-540 respectively. Additionally, these residues are flanked by catalytic residues analogous to Arg-38, Trp-41, Asp-208 and Trp-179 of the pea cytosolic APX. The two domains of *Euglena* APX also contained Cys-32 of the pea APX, which is situated close to the AsA-binding site and is conserved in all known isoforms of APX [2,3]. Arg-172 of pea APX plays an important role in the utilization of AsA to form Compound II [27]. Moreover, the crystal structure of the soybean APX and AsA complex indicates that Lys-30 contributes to the substrate binding [24]. These residues are conserved in both APX-N and APX-C of *Euglena* APX. The sequences also contained the metal-binding five-residue K$^+$ site (comprising Asp-187, Thr-164, Thr-180, Asn-182 and Ile-185) [28], although Ile-185 was replaced by a lysine residue and a valine residue in APX-N and APX-C respectively. A comparison of cytosolic APX from soybean with cytochrome c peroxidase showed that APX does not contain an additional loop structure which blocks the AsA-binding site of cytochrome c peroxidase (residues 34–41) [24]. The sequence alignment (Figure 1) and structure model (Figure 3) indicated that the loop structure of *Euglena* APX was missing in both the APX-N and APX-C domains, as well as in cytosolic APX from soybean and pea. Therefore, consistent with the idea that the truncated recombinant enzymes would be catalytically active, we found the kinetics of the recombinant APX-N and APX-C to be comparable with that of the plant cytosolic APX and the native *Euglena* APX with AsA and H$_2$O$_2$ (Table 1). However, in contrast with the native enzyme, the recombinant APX-N and APX-C domains did not show any activity towards alkyl hydroperoxide (Table 1). On the other hand, the recombinant FL-APX exhibited catalytic activity towards alkyl hydroperoxide, like the native enzyme, indicating that the intramolecular dimeric structure is correlated with substrate recognition. Badyal et al. [29] have recently reported that cytosolic APX potentially has a catalytic role.

![Figure 7](image-url) Suppressing APX expression promotes H$_2$O$_2$ accumulation in *Euglena* cells

(A) RT–PCR analysis of APX and EF1-α (for normalization) mRNA levels using total RNA from Euglena cells transfected with (+ dsRNA) and without (− dsRNA) dsRNA. Fragments corresponding to APX and EF1-α were amplified by PCR using the cDNA preparations as templates. (B) APX activity in crude extracts prepared from Euglena cells. (C) Detection of cellular H$_2$O$_2$ by fluorescence microscopy. Cellular H$_2$O$_2$ was measured using a H$_2$O$_2$-specific fluorescence probe, BES-H$_2$O$_2$-Ac. Cultures of representative cells were photographed 1 week after the dsRNA was introduced.
binding ability with t-butyl hydroperoxide, but does not form the Compound I intermediate. Although it is difficult to explain the mechanism for substrate recognition for Euglena APX, the conformational alteration of the mature APX by the formation of the intramolecular dimer may facilitate its activity toward alkyl hydroperoxide.

In Euglenoids, trans-splicing has been described as a mechanism for the generation of mature mRNAs [30]. It is apparent that the 5′ end of mRNA is replaced by a short spliced leader exon from a small spliced leader RNA, as can be seen at the 5′ end of the full-length cDNA of Euglena APX (Supplementary Figure S1). We suspected that the mature APX mRNA, except its 5′ end, was generated by trans-splicing or cis-splicing machinery. A genomic Southern analysis using individual probes from APX-N and APX-C produced similar signalling patterns (Figure 5). In addition, a partial analysis of the genomic sequence indicated that both domains definitely exist in the same segment of the gene (Supplementary Figure S3). The border sequences of exon/introns in APX-N and APX-C did not follow the canonical GT/AG rule, but non-conventional introns have been observed in other genes in Euglena [31–33]. Accordingly, we concluded that the APX mRNA is generated by a cis-splicing mechanism. Presumably, Euglena has incidentally acquired its unique APX gene through the tandem repetition of a nearly identical sequence during evolution.

Surprisingly, the primary structure of Euglena APX contained an N-terminal extension of 102 amino acid residues, which was very similar to a plastid signal of class II-type in Euglena (Supplementary Figure S2; [21]), although the mature protein was localized to the cytosol (Figure 4). As far as we know, this is the first case of a protein that requires processing in order to distribute in the cytosol. It is worth considering a route via the ER (endoplasmic reticulum) for transporting APX into the cytosol, because this kind of signal in Euglena, unlike other known photosynthesizing organisms, utilizes a transport vesicle and a hydrophobic region in the signal sequence acts as a stop transfer sequence to prevent complete transfer into the ER, so that the mature protein remains in the plastid [34,35].

**Contribution of APX to H2O2 metabolism in Euglena cells**

The suppression of APX expression by RNAi indicated that Euglena APX physiologically contributes to the metabolism of H2O2 in cells (Figure 7). We demonstrated previously that Euglena cells produce H2O2 during ordinary metabolic processes and electron transport in plasts and mitochondria [36]. In the case of higher plants, APX isoenzymes distributed in plasts, the cytosol and microbodies play a key role in the metabolism of H2O2 at the site of its generation [1,2]. It is of interest that the Euglena APX is present only in the cytosol and leads us to question how it manages the intracellular level of H2O2 in various cellular compartments. Plasts are the major site for the generation of H2O2 due to an abundance of O2 produced by photochemical reactions and concomitant photosynthetic electron transport. Photosynthesis in higher plants is highly susceptible to H2O2, but that in Euglena is not due to the resistance of the fucoxanthin-6,16-sedoheptulose-1,7-bisphosphate, NADP+–glyceraldehyde-3-phosphate dehydrogenase and ribulose-5-phosphate kinase enzymes of the Calvin cycle to H2O2 at up to 1 mM [37]. Furthermore, H2O2 generated in both plasts and mitochondria in Euglena cells diffused into the cytosol, where it was decomposed by APX [36]. Taken together, H2O2-tolerance and -diffusion systems in Euglena cells would help to allow the cytosolic APX to manage the level of cellular H2O2. We have shown previously that the expression of Euglena APX is post-transcriptionally regulated during the development of proplasts into mature functional chloroplasts in response to light illumination, also supporting its role in the regulation of H2O2 generated in the chloroplasts [19].

In higher plants, the cytosolic APX has many subtle functions in controlling cellular redox conditions. Among various APX isoenzymes, the cytosolic form is known to be highly responsive to oxidative stresses, including high-light exposure [38,39]. A previous transcriptomic analysis also indicated the susceptibility of the cytosolic APX to environmental stress [40]. Furthermore, an Arabidopsis mutant lacking APX1, one of the typical cytosolic isoenzymes, showed a significant increase in cellular H2O2, and an up-regulation of the expression of various genes, including those in response to stress [41,42]. We reported previously that suppression of a cytosolic APX by gene silencing in tobacco BY-2 cells resulted in a significant increase in cellular H2O2, and cross-tolerance to heat and salinity stress, accompanied by the activation of a protein kinase and the up-regulation of stress-responsive gene expression [43]. Thus the cytosolic APX in higher plants is emerging as a key enzyme for the redox gene network via H2O2 metabolism. Although the redox regulation of cellular function including gene expression in eukaryotic algae is still largely unknown, the silencing of cytosolic APX in Euglena will provide a clue as to the redox signalling of eukaryotic algae.

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SUPPLEMENTARY ONLINE DATA

Euglena gracilis ascorbate peroxidase forms an intramolecular dimeric structure: its unique molecular characterization

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Figure S1 Full-length cDNA sequence of Euglena APX

The amino acid sequences deduced from the ORF are shown below the nucleotide sequences. The amino acid sequences of N-terminal and proteolytic peptides of purified Euglena APX are underlined (reference [13] in the main paper). The box indicates the putative linker region between the APX-N and APX-C domains. The double underline shows the spliced leader sequence. The arrowhead indicates the cleavage site of the N-terminal leader sequence. The distal and proximal histidine residues are shown by H.

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Figure S2 Scatter plot showing TMHMM probability for the class II targeting signal of the Euglena plastid

Potential membrane-spanning regions were identified using the TMHMM program (reference [20a] in the main paper). The cluster ID numbers for proteins analysed are as follows: PsbW, ELL00005545; PsaE, ELL0000098; phosphoribulokinase, ELL0000240; peptide chain release factor 2, ELL00001542; chloroplast 50S ribosomal protein L15, ELL0001392. The N-terminal extension of 102 amino acid residues of Euglena APX shows the presence of the hydrophobic region associated with the common signal sequence in all class II proteins in Euglena. TMH1, transmembrane helix 1.

Figure S3 Partial sequence of the Euglena APX gene amplified with primers extending over APX-N and APX-C regions

Non-coding regions are in lower case. Arrows indicates the primer sequences used for the amplification. The boxes show the border sequence of exon/introns.