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Regulation of glyceraldehyde-3-phosphate dehydrogenase in the eustigmatophyte *Pseudocharaciopsis ovalis* is intermediate between a chlorophyte and a diatom

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Running title: Regulation of GAPDH in a eustigmatophyte

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Abbreviations:

CP12, chloroplast protein; DTT, dithiothreitol; BPGA, 1,3-bisphosphoglyceric acid; ox, oxidised; red, reduced; GAPDH, EC 1.2.1.13 glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$) (phosphorylating); NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; PRK, EC 2.7.1.19 phosphoribulokinase.

Abstract
The regulation of NADPH-dependent GAPDH was analysed in the chromalveolate (eustigmatophyte) *Pseudocharaciopsis ovalis* and compared to the well-studied chlorophyte *Chlamydomonas reinhardtii* and to another chromalveolate (bacillariophyte), *Asterionella formosa.* Optimal pH for GAPDH activity in *P. ovalis* and *C. reinhardtii* ranged between 8 and 9 but in *A. formosa*, ranged between 6.2 and 8.1. Assuming dark pH values of about 7 in the plastids of all three species, GAPDH would be down-regulated in the dark in *C. reinhardtii* and *P. ovalis*, but fully-active in *A. formosa*. The time required for half-maximal GAPDH activity on transfer to reducing conditions, was significantly different in each species: 1.4, 4.0 and 5.9 mins in *A. formosa, P. ovalis* and *C. reinhardtii* respectively. Under oxidised conditions in *P. ovalis* and *A. formosa,* NADPH caused a large inhibition in GAPDH activity even at very low concentrations (10 to 20 µM) unlike in *C. reinhardtii*. This inhibition was relieved by addition of a reducing agent suggesting that NADPH could control GAPDH activity under dark-light transitions. A small increase of GAPDH activity with NADP at concentrations higher than 0.5 mM was observed with *P. ovalis* and *C. reinhardtii*, while a greater than 1.5-fold stimulation was observed in *A. formosa*. Regulation of GAPDH in *P. ovalis* was intermediate between the diatom and the chlorophyte and the possible evolutionary reasons for this are discussed.

Key words: *Asterionella formosa*, Calvin cycle, *Chlamydomonas reinhardtii*, CP12, enzyme regulation, regulation by pH

Introduction

The Benson-Bassham-Calvin cycle, responsible for CO_2 assimilation, is regulated by light enabling the cells to switch between dark and light metabolism. Enzyme regulation in higher plants and green algae can result from changes in the supply of light energy that alters the redox state, pH, Mg^{2+} and metabolite concentrations within the plastid (Heldt *et al.*, 1973; Werdan *et al.*, 1975; Portis & Heldt, 1976; Buchanan, 1980; Schurmann & Jacquot, 2000) and also from protein-protein interaction (Gontero *et al.*, 2001). Two key chloroplast enzymes, phosphoribulokinase (PRK, EC 2.7.1.19) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.13), use the products of the light-reactions and are regulated by dark-light transitions. PRK catalyses the phosphorylation of ribulose-5-phosphate with ATP to generate ribulose-1,5-bisphosphate and GAPDH catalyses the reductive dephosphorylation of 1,3-bisphosphoglycerate, using NADPH preferentially, but also NADH, to generate glyceraldehyde-3-phosphate, NAD(P) and inorganic phosphate.

The chloroplast GAPDH exists in several isoforms. In cyanobacteria, green and red algae and higher plants, GAPDH is composed of four identical GapA subunits (A_4) (Petersen *et al.*, 2006; Trost *et al.*, 2006). In higher plants, in addition to GapA, there is a heterotetramer composed of two GapA and two GapB subunits (A_2B_2) and a hexadecamer (A_8B_8) (Baalmann *et al.*, 1994; Baalmann *et al.*, 1995). GapA and GapB subunits are very similar, but the GapB subunit contains a regulatory C-terminal extension of about 30 residues containing two cysteine residues that are the target of thioredoxins (Cerff, 1979; Brinkmann *et al.*, 1989; Sparla *et al.*, 2002). The GapA/B subunits have not been found in diatoms (chromalveolates). Instead, chromalveolates contain enzymes that derive from the GapC glycolytic isoform: a glycolytic GapC2 specifically using NADH and a chloroplast GapC1 lacking the regulatory cysteine residues (Liaud *et al.*, 1997; Takishita *et al.*, 2009).

The isoforms of GAPDH, such as GapA and GapC1, that do not possess regulatory cysteine residues may still be redox-regulated by interacting with other proteins (Boggetto *et al.*, 2007; Erales *et al.*, 2008; Maberly *et al.*, 2010). Specifically, GAPDH can form a supramolecular complex with PRK via an intrinsically disordered protein of 8.5 kDa, CP12 (Pohlmeyer *et al.*, 1996). CP12 has redox-sensitive cysteine residues that confer regulatory control to GAPDH. It is widespread and has been found in many photosynthetic organisms (Groben *et al.*, 2010). The well-studied PRK/GAPDH/CP12 supramolecular complex in higher plants and green algae, can be dissociated under conditions mimicking light. *In vitro*, a reducing agent, dithiothreitol (DTT) mimicking thioredoxin, dissociates the supramolecular complex and the released enzymes become active. Moreover, metabolites such as NADPH and NADP can also regulate GAPDH activity in photosynthetic organisms. In higher plants, NADPH in the presence of DTT has been shown to stimulate enzyme activity, also via the depolymerisation of a high molecular weight form of GAPDH (Pupillo & Piccari, 1973; Lazaro *et al.*, 1986; Nicholson *et al.*, 1987).

Most of the knowledge about Calvin cycle regulation has been obtained from higher plants and, within the eukaryotic algae, the chlorophyte *Chlamydomonas reinhardtii* P.A Dangeard. However, aquatic productivity largely results from photosynthesis by other groups of algae. Regulation of GAPDH and PRK in the diatom *Asterionella formosa* Hassall is different (Boggetto *et al.*, 2007; Erales *et al.*, 2008) and studies on a range of species showed a relationship between their regulation and their phylogeny in most species (Maberly *et al.*, 2010). Specifically, the eustigmatophyte *Pseudocharaciopsis ovalis* (Chodat) Hibberd, unexpectedly, had properties closer to the Plantae than to the chromalveolates to which it belongs (Maberly *et al.*, 2010). It has been pointed out that modern mechanistic studies have largely overlooked groups such as the eustigmatophytes (Green, 2011). The aim of this work was therefore to analyse, in detail, the regulation of GAPDH in a eustigmatophyte,

Pseudocharaciopsis ovalis (Chodat) Hibberd, and a diatom, *A. formosa* (both chromalveolates) and to compare these results with the well-studied green alga *C. reinhardtii* (Plantae).

Materials and methods

Phytoplankton cultivation and harvesting

All cultures were typically grown in 1.5 L batch cultures under a 14:10 h (light:dark) regime at about 65 µmol m^{-2} s^{-1} (photosynthetically active radiation; 400 – 700 nm). *Asterionella formosa* RGC028 (Boggetto *et al.*, 2007) was grown at 16.5°C, under agitation in Diatom Medium (Beakes *et al.*, 1988) but with twice the concentration of vitamins. *Chlamydomonas reinhardtii* ($ST4^{+}$) cells were grown mixotrophically at 23°C as previously described (Avilan *et al.*, 1997). *Pseudocharaciopsis ovalis* CCAP 809/3 was grown at 23°C in Modified Woods Hole Medium (http://www.ccap.ac.uk/media/documents/MWC.pdf).

Extraction of proteins

Dense cultures were harvested, either after 48 hours in the dark or in the middle of the photoperiod, by centrifugation (3750 g for 15 min at 4°C, Allegra X-15R Benchtop Centrifuge, Beckman Coulter Inc). Cell pellets were subsequently resuspended in a minimum volume of 15 mM Tris, 4 mM EDTA, 10% glycerol, and in the presence of a cocktail of protease inhibitors (0.5 µg mL^{-1}, Sigma Inc., Saint Louis, MO, USA) at pH 7.9 on ice either with (i) no addition (control) or supplemented with (ii) 0.1 mM NAD, (iii) 0.1 mM NADP, or (iv) 0.1 mM NADPH as indicated. The resulting suspensions were broken using an ultrasonicator (Sonics & Materials Inc, Vibracell, Bioblock, Danbury, Connecticut, USA). The broken cells were subsequently centrifuged at 27,000 g for 25 min at 4°C. The supernatant, containing the crude extract, was kept on ice after adding glycerol to a final concentration of 10%. Protein concentration in all crude extracts was assayed with the Bio-

Rad (Hercules, CA, USA) protein dye reagent, using bovine serum albumin as a standard (Bradford, 1976).

Measurement of enzyme activity

Extracts for NAPDH-GAPDH assays were incubated at room temperature for 20 minutes before measurement under the following conditions: (i) without addition (untreated), or with (ii) 20 mM reduced dithiothreitol (red DTT) or (iii) 25 mM oxidized dithiothreitol (ox DTT). Aliquots from the different incubation mixtures were withdrawn and the activity of GAPDH was measured with 0.2 mM NADPH as cofactor plus 1 mM 1,3-bisphosphoglyceric acid (BPGA) synthesized according to (Graciet *et al.*, 2003). In experiments where GAPDH was oxidized by incubation with oxidized DTT, reactivation was checked by adding 10 mM reduced DTT to the same cuvette, a concentration that exceeded the concentration of oxidized DTT in the cuvette (0.75 mM). The GAPDH activity was followed by measuring the change in absorbance of NADPH at 340 nm using a UV/Visible Lambda 25 double-beam Perkin Elmer spectrophotometer (Courtaboeuf, France). Activity was measured at least four times and was normalised to a protein basis. All reagents for enzyme activity measurements were from Sigma Inc.

Kinetic analysis

The NADPH dependent activity of GAPDH incubated with 20 mM DTT was followed as a function of incubation time for all three species. The curves were fitted with Sigma Plot software (v 10.0, Systat Software GmbH, Erkrath, Germany) to experimental data using the following equation:

$$Q = A^{*}\left(1\text{-}e^{-\lambda^{*}\,t}\right) + c \qquad (1)$$

where Q is the rate of the reaction per mg of protein, A and λ are the amplitude and the time constant of the activation process, respectively and c is the reaction rate in the absence of DTT in the incubation. Rates were expressed as a percentage of the maximal rate, A plus c.

NADPH-dependent activity of GAPDH at different pH values

NADPH-dependent activity of GAPDH from the three species was measured at pH values ranging from 6.2 to 9.5; at least three measurements were performed for each species at each pH. Three buffers were used: 50 mM MES/KOH for pH 6–6.8, 50 mM HEPES/KOH for pH 6.8–7.5 and 50 mM glycylglycine for pH 7.5–9.5. The remaining components were as follows: 50 mM KCl, 15 mM $MgCl_2$, 0.5 mM EDTA and 5 mM DTT. Activity of GAPDH was also measured with or without 5 mM DTT after incubation with 1 mM NADPH for *A. formosa* and *P. ovalis* at four pH values from 6 to 8.1.

Effect of NADP(H) concentration on NADPH- GAPDH

Cells were harvested during the light, broken and GAPDH from the three species was incubated with varied NADPH concentrations from 0 to 1 mM for 25 minutes and then aliquots were removed and activity measured as described above. Reduced DTT at a final concentration of 20 mM was added to the cuvettes to check if the activity could be recovered. Similar experiments were also performed using a range of concentrations from 0 to 2 mM NADP.

Statistical analysis

Variance ratio tests (F-test) were performed to determine if the kinetics fits were significantly different from one another (Mead & Curnow, 1983). Student's t-tests were performed in

Microsoft Excel to determine the significance of different GAPDH activity in cells from various treatments.

Results

pH profiles of GAPDH

The NADPH-dependent activity of GAPDH in the three species was tested at a range of pH values. There was a marked difference in GAPDH activity as a function of pH between *A. formosa* and the two other species (Fig. 1). The pH range where activity exceeded an arbitrary 75% of maximal activity was between 6.2 and 8.1 in *A. formosa*, 7.6 and 9.0 in *P. ovalis* and 7.7 and 9.5 in *C. reinhardtii*. The pH pattern of GAPDH activity in the chromalveolate *P. ovalis* was thus more similar to the green alga, *C. reinhardtii*, than to the other chromalveolate tested, *A. formosa*. In the subsequent experiments, activity was measured at pH 7.7 which was appropriate for all three species.

FIGURE 1

Rate of activation of GAPDH by DTT

Kinetic analyses were performed to investigate the rate of GAPDH-activation by DTT (Fig. 2). Curve-fitting analyses showed that the time required for half-maximal activity was 1.4 ±0.3 min (mean±SD) in *A. formosa*, 4.0±1.3 min in *P. ovalis* and 5.9±0.7 min in *C. reinhardtii*. Variance ratio tests on the data expressed as percent activity showed that the half-time of activation for each species was significantly distinct ($P=0.011$ to $P<0.001$). Under these conditions, the maximal activity of GAPDH was 594±31, 160±20 and 1180±70 nmol min^{-1} mg $protein^{-1}$ for *A. formosa*, *P. ovalis* and *C. reinhardtii*, respectively.

FIGURE 2

Redox regulation of NADPH-GAPDH in cells from dark and light

The NADPH-dependent activity of GAPDH from cells of *P. ovalis*, harvested after a dark- or light-period (hereafter designated 'dark cells' and 'light cells') and broken in the presence of different nicotinamide nucleotides (0.1 mM), was measured without treatment or after incubation with either reduced or oxidised DTT (Fig. 3).

Broadly, the results showed that dark cells were in an oxidised state and light cells were in a reduced state. For the control from the dark cells, the rate of the untreated sample was not significantly different from the rate of the oxidised sample, but was lower than the rate of the reduced sample. The reducing treatment caused an activation by a factor of 1.8 ± 0.2. For the controls from the light cells, the rate of the untreated sample was slightly, but significantly lower (1.26 ± 0.15) than the rate of the reduced sample but was significantly higher than the rate of the oxidised sample. The oxidising treatment caused an inhibition of 5.7 ± 1 for the controls from the light cells. For the four untreated samples, rates in the light cells were significantly higher than those in the dark cells, apart from the sample containing NAD, by a factor of between 1.5 and 2.2 (Fig. 3). A similar comparison for the oxidised samples showed that the rates of the light cells were not significantly different from the rates in the dark cells except for the sample with NAD that was lower in the light (Student's t-test, $P<0.05$). Overall, these results suggest that redox-regulation of GAPDH in *P. ovalis* occurs in the light and dark cells *in vivo* and that DTT mimics *in vitro* the redox-changes that occur *in vivo*.

The effect of NAD, NADP and NADPH on NADPH-GAPDH

In the dark cells, in all samples that contained NAD and NADP, rates were not significantly different from the control, while NADPH resulted in a decrease of activity in untreated and

reduced cells (Fig. 3). However, the rate of the dark cells that contained NADPH and were reduced, was significantly higher, by 2.4- and 2.3-fold, than the oxidised or untreated sample (Student's t-test; $P<0.01$ and $P<0.05$, respectively). In the light cells, the untreated NADPH sample was also slightly (1.3 ± 0.13) but significantly (Student's t-test; $P<0.05$) lower than the control. This effect was not observed in reduced conditions (Fig. 3). In the light cells, the activity of the oxidised sample containing NADP was significantly higher (Student's t-test; $P<0.001$) than all other oxidised samples and was similar to that of the untreated sample. This result suggests that NADP might prevent oxidation in the light.

FIGURE 3

Inhibition of NADPH-GAPDH by [NADPH] and its pH dependency. The results in Figure 3 showed that incubation of the samples with 0.1 mM NADPH affected the reaction rates of NADPH-GAPDH. Since NADPH can regulate activity of GAPDH in other photosynthetic organisms, GAPDH activity was measured after incubation with NADPH at a range of concentrations in all three species.

For *P. ovalis* and *A. formosa*, there was a dramatic reduction in NADPH-GAPDH activity even at low concentrations of NADPH: at 10 µM NADPH, activity was about 40 % of the maximum in both species (Fig. 4A and B). At all concentrations of NADPH, there was a full recovery of activity when DTT was added to the incubation mixture (Fig. 4, triangles). Furthermore, the recovery was immediate since addition of DTT to the measuring cuvette gave the same result (data not shown). In contrast to the two chromalveolates, NADPH had no effect on GAPDH from *C. reinhardtii* using this range of concentrations (Fig.4C). By removing the GAPDH substrate, BPGA, from the assay, we showed that the residual activity

(about 20% of maximum) at high NADPH concentration was not caused by contaminating activity of a NADPH oxido-reductase.

FIGURE 4

The percent inhibition by 1 mM NADPH increased with decreasing pH in a similar way in both *A. formosa* and *P. ovalis* (Fig. 5) despite their GAPDH pH-profiles being different (Fig. 1). The samples incubated with 1 mM NADPH had low activity that was independent of pH (data not shown). Therefore, the changing inhibition resulted from changes in maximal activity and low, pH-independent, activity in the presence of 1 mM NADPH.

FIGURE 5

Activation of NADPH-GAPDH by NADP. The effect of NADP, the product of the GAPDH reaction, was measured in all three species. At concentrations lower than 0.5 mM, NADP slightly inhibited GAPDH activity in *P. ovalis* and in *A. formosa* but had no significant effect on *C. reinhardti* (Fig. 6). At concentrations higher than 0.5 mM, the pattern of regulation in the three species was different with a small activation in *P. ovalis* and a large activation in *A. formosa* while GAPDH from *C. reinhardtii* was only slightly activated at 2 mM NADP (Fig. 6).

FIGURE 6

Discussion

In order to optimise photosynthesis, the supply of reducing power and ATP from the light reactions, has to be fine-tuned with the demand for these products during carbon-fixation

(Halliwell, 1984). Conversely, in the dark, reducing power and ATP needs to be directed towards other metabolic pathways. Enzyme regulation in higher plants and green algae has been extensively studied and been shown to act via a range of factors including pH, metabolites, redox and protein-protein interactions (Gontero *et al.*, 2006). Much less is known for chromalveolates (Michels *et al.*, 2005; Boggetto *et al.*, 2007; Erales *et al.*, 2008; Maberly *et al.*, 2010) and we present here for the first time a detailed study of GAPDH regulation in a eustigmatophyte.

Regulation by pH

The pH in the stroma of higher plants and green algae is about 7 in the dark and 8 in the light (Heldt *et al.*, 1973; Werdan *et al.*, 1975). In *C. reinhardtii*, the activity of GAPDH at pH 7 was only one-third of that at pH 8.5, so the lower pH in dark chloroplasts may partly-down regulate activity. In diatoms, the intracellular pH values in light and dark have been determined in two species. The internal pH was 7.44 and 7.54 in the light and 6.94 and 7.05 in the dark in *Phaeodactylum tricornutum* and *Cyclotella* sp., respectively (Coleman & Rotatore, 1995), and was thus slightly lower than in *C. reinhardtii* and higher plants, assuming that this also reflects chloroplast pH. The pattern of response of GAPDH to pH found here for the diatom *A. formosa* suggests that at pH values in the dark, pH 6.9 to 7.1, the enzyme would be fully-activated. Thus, pH may not modulate GAPDH activity in diatoms. In contrast, the response of GAPDH to pH in the eustigmatophyte *P. ovalis* is similar to that in the chlorophyte *C. reinhardtii* so, if chloroplast pH is in the same range as in other photosynthetic organisms, GAPDH could also be partly-regulated by pH during light-dark transitions in this species.

Redox regulation

Redox regulation via the ferredoxin/thioredoxin system plays an important rôle in controlling CO_2 assimilation in higher plants and green algae but is thought to play a minor rôle in diatoms. However, many thioredoxins are targeted into the diatom plastid (Weber *et al.*, 2009) and GAPDH in *A. formosa* is regulated by DTT (Boggetto *et al.*, 2007; Erales *et al.*, 2008; Maberly *et al.*, 2010) indicating that it could be a possible target for thioredoxins. We found here that the activation of GAPDH by DTT was rapid in the three species (between 1.4 and 6 minutes), and fast enough to be ecophysiologically significant. The half-time of GAPDH activation in *P. ovalis* was intermediate between *A. formosa* and *C. reinhardtii.* In *P. ovalis,* the activity in light cells was higher than that of dark cells and sensitive to oxidation, not reduction, while the activity in dark cells was only sensitive to reduction and not oxidation. This is therefore consistent with redox-regulation of GAPDH activity of *P. ovalis, in vivo.* It also suggests that DTT, as in higher plants and green algae (Baier & Latzko, 1975; Zimmermann *et al.*, 1976), can mimic the redox-consequences of light and dark.

Regulation by nicotinamide nucleotides

Metabolites can also regulate enzyme activity. In higher plants, pre-incubation with NADPH or NADP causes GAPDH activity to increase (O'Brien *et al.*, 1977). This activation results from the dissociation of a less active, high molecular weight form of GAPDH into a more active tetrameric form (Wolosiuk & Buchanan, 1976; Baalmann *et al.*, 1994; Baalmann *et al.*, 1995). A similar but small increase of activity with NADP at concentrations higher than 0.5 mM was observed with *P. ovalis* and *C. reinhardtii*, while a greater than 1.5-fold stimulation was observed in *A. formosa*. Concentrations of NADP in chromalveolates are unknown but in higher plants concentrations of 0.5 and 0.6 mM have been reported in dark and light, respectively (Heineke *et al.*, 1991), so NADP could be a positive-regulator of GAPDH activity.

In contrast to higher plants, our data showed that GAPDH from the eustigmatophyte *P. ovalis* and the diatom *A. formosa*, but not from the green alga *C. reinhardtii*, was strongly deactivated by NADPH in the oxidizing conditions that mimic the dark state of the chloroplast. Though in chromalveolates, the NADPH concentration is unknown, in chloroplasts from spinach it has been reported to be between 0.3 and 0.5 mM in the light and 0.12 mM in the dark (Cseke *et al.*, 1982) (Heineke *et al.*, 1991). In the cyanobacterium, *Synechococcus* PCC7942, Tamoi *et al*. (Tamoi *et al.*, 2005) reported similar NAPDH concentrations of 0.37 mM in the light and 0.31 mM in the dark. If this range of NADPH concentration is present in chromalveolate plastids in the dark it would effectively inhibit GAPDH because we found inhibition even at 10 to 20 µM NADPH in both *P. ovalis* and *A. formosa*.

Down-regulation of GAPDH by its substrate, NADPH, may be a mechanism to switch-off the Calvin cycle in the dark and direct the use of NADPH towards other pathways such as the biosynthesis of fatty acids and isoprenoids such as fucoxanthin, and reducing power for nitrite reduction that also occurs in the plastid (Wittpoth *et al.*, 1998; Wilhelm *et al.*, 2006). The presence or absence of the oxidative pentose pathway that produces NADPH, is unknown in *P. ovalis*, but this cycle is absent at least from the diatom plastid (Michels *et al.*, 2005). Therefore if NADPH becomes limiting this regulation will avoid futile cycling (Wilhelm *et al.*, 2006; Kroth *et al.*, 2008). The apparent lack of down-regulation of GAPDH by pH in the dark in *A. formosa* may be another reason for the observed inhibition by NADPH. The inhibition of GAPDH by NADPH in the two chromalveolates occurs at all pH values but can be reversed by adding a reducing agent that "switches on" the activity of GAPDH. Thus, the modulation of activity of GAPDH by NADPH could be a way to control the activity of this enzyme under dark-light transitions but whether this effect is direct or

indirect (through protein-protein interactions existing in the extract) has still to be determined.

Phylogenetic diversity in GAPDH regulation

The three different algae studied here are members of two phylogenetic supergroups, the chromalveolates and the Plantae, that together are responsible for the majority of global primary productivity (Falkowski *et al.*, 2004). These two groups differ in the isoform of GAPDH that they have and this translates to regulatory control of this enzyme. Inhibition of GAPDH by NADPH has not been observed in *C. reinhardtii* or higher plants with the GapA or GapB forms of GAPDH. In contrast, inhibition of GAPDH by NADPH occurs in the two chromalveolates which have the GapC1 isoform. This isoform is believed to have evolved from a glycolytic enzyme derived from proteobacteria (Liaud *et al.*, 1997). In its glycolytic mode of action, inhibition by NADPH, the product of the reaction, would prevent futile cycling. One possibility is that this inhibition has been retained in the chloroplast form of GapC1 that performs the reverse reaction.

The Eustigmatophyta was first recognised as a separate taxonomic group in 1971 (Hibberd & Leedale, 1971), and are members of the Heterokonta, within the chromalveolates, that contains many algal species. Based on molecular evidence, Takishita *et al*. (Takishita *et al.*, 2009) showed that the GAPDH sequence in another eustigmatophyte, *Nannochloropsis oculata*, was closely-related to diatoms and chrysophytes. However, the regulation of GAPDH in *P. ovalis*, was intermediate between *C. reinhardtii* and *A. formosa.* It was similar to *C. reinhardtii* in its pH-profile and stimulation by NADP, intermediate between *C. reinhardtii* and *A. formosa* in its rate of activation by reducing agent, and similar to *A. formosa* as regards to its inhibition by NADPH. Differences in GAPDH regulation between *P. ovalis* and *A. formosa* may result from metabolic costs and benefits that play-out in the

habitat that each species inhabits. In addition, recent analyses of genetic sequences have suggested that diatoms and other chromalveolates may have both red and green alga ancestors (Moustafa *et al.*, 2009). It is possible that the extent of horizontal gene transfer has varied among groups of algae, and that this could be the cause of the different pattern of GAPDH regulation between *P. ovalis* and *A. fomosa* reported here and other chromalveolates examined in a broad screen (Maberly *et al.*, 2010). However, it is maybe more likely that the effects may result from interactions with other proteins such as CP12 and/ or the formation of supramolecular complexes. These hypotheses are currently being tested using purified protein from *A. formosa*.

Eustigmatophytes have been poorly studied (Green, 2011) and clearly, there is a gap in the knowledge of these and other eukaryotic algae that in total contribute around half of global primary productivity (Field *et al.*, 1998). This report is a first step towards pin-pointing the precise mechanisms that control enzyme regulation in a eustigmatophyte and another chromalveolate.

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References

AVILAN, L., GONTERO, B., LEBRETON, S., & RICARD, J. (1997). Memory and imprinting effects in multienzyme complexes--I. Isolation, dissociation, and reassociation of a phosphoribulokinase-glyceraldehyde-3-phosphate dehydrogenase complex from *Chlamydomonas reinhardtii* chloroplasts. *Eur J Biochem* **246**(1): 78-84.

BAALMANN, E., BACKHAUSEN, J.E., KITZMANN, C., & SCHEIBE, R. (1994). Regulation of NADP-dependent glyceraldehyde 3-phosphate dehydrogenase activity in spinach chloroplast. *Botanica Acta* **107**: 313-320.

BAALMANN, E., BACKHAUSEN, J.E., RAK, C., VETTER, S., & SCHEIBE, R. (1995). Reductive modification and nonreductive activation of purified spinach chloroplast NADP-dependent glyceraldehyde-3-phosphate dehydrogenase. *Arch Biochem Biophys* **324**(2): 201-208.

BAIER, D. & LATZKO, E. (1975). Properties and regulation of C-1-fructose-1,6-diphosphatase from spinach chloroplasts. *Biochim Biophys Acta* **396**(1): 141-148.

BEAKES, G., CANTER, H.M., & JAWORSKI, G.H.M. (1988). Zoospores ultrastructure of *Zygorhizidium affluens* and *Z. planktonicum*, two chytrids parasitizing the diatom *Asterionella formosa*. . *Can J Bot* **66**: 1054-1067.

BOGGETTO, N., GONTERO, B., & MABERLY, S.C. (2007). Regulation of phosphoribulokinase and glyceraldehyde 3-phosphate dehydrogenase in a freshwater diatom, *Asterionella formosa* (Bacillariophyceae). *Journal of Phycology* **43**(6): 1227-1234.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.

BRINKMANN, H., CERFF, R., SALOMON, M., & SOLL, J. (1989). Cloning and sequence analysis of cDNAs encoding the cytosolic precursors of subunits GapA and GapB of chloroplast glyceraldehyde-3- phosphate dehydrogenase from pea and spinach. *Plant Mol Biol* **13**(1): 81-94.

BUCHANAN, B.B. (1980). Role of light in the regulation of chloroplast enzymes. *Annu Rev Plant Physiol* **31**: 341-374.

CERFF, R. (1979). Quaternary structure of higher plant glyceraldehyde-3-phosphate dehydrogenases. *Eur J Biochem* **94**(1): 243-247.

COLEMAN, J.R. & ROTATORE, C. (1995). Photosynthetic inorganic carbon uptake and accumulatiob in two marine diatoms. *Plant , Cell and Environment* **18**: 918-624.

CSEKE, C., NISHIZAWA, A.N., & BUCHANAN, B.B. (1982). Modulation of chloroplast phosphofructokinase by NADPH : a mechanism for linking light to the regulation of glycolysis. *Plant Physiol* **70**(3): 658-661.

ERALES, J., GONTERO, B., & MABERLY, S.C. (2008). Specificity and function of glyceraldehyde-3-phosphate dehydrogenase in a freshwater diatom, *Asterionella formosa* (BACILLARIOPHYCEAE). *Journal of Phycology* **44**(6): 1455-1464.

FALKOWSKI, P.G., KATZ, M.E., KNOLL, A.H., QUIGG, A., RAVEN, J.A., SCHOFIELD, O., & TAYLOR, F.J. (2004). The evolution of modern eukaryotic phytoplankton. *Science* **305**(5682): 354-360.

FIELD, C.B., BEHRENFELD, M.J., RANDERSON, J.T., & FALKOWSKI, P. (1998). Primary production of the biosphere: integrating terrestrial and oceanic components. *Science* **281**(5374): 237-240.

GONTERO, B., AVILAN, L., & LEBRETON, S. (2006). Control of carbon fixation in chloroplasts. In *Annual Plant Reviews* (Plaxton, W.C., McManus, M.T., editor), 187-218. Blackwell Publishing Oxford.

GONTERO, B., LEBRETON, S., & GRACIET, E. (2001). Multienzyme complexes involved in the Benson-Calvin cycle and in fatty acid metabolism. In *Annual Plant Reviews* (McManus, M.T., Laing, W.A., Allan, A.C., editor), 120-150. Sheffield Academic Press, Sheffield.

GRACIET, E., LEBRETON, S., CAMADRO, J.M., & GONTERO, B. (2003). Characterization of native and recombinant A4 glyceraldehyde 3- phosphate dehydrogenase. *European Journal of Biochemistry* **270**(1): 129-136.

GREEN, B.R. (2011). After the primary endosymbiosis: an update on the chromalveolate hypothesis and the origins of algae with Chl c. *Photosynth Res* **107**(1): 103-115.

GROBEN, R., KALOUDAS, D., RAINES, C.A., OFFMANN, B., MABERLY, S.C., & GONTERO, B. (2010). Comparative sequence analysis of CP12, a small protein involved in the formation of a Calvin cycle complex in photosynthetic organisms. *Photosynth Res* **103**(3): 183-194.

HALLIWELL, B. (ed.) (1984). *Chloroplast metabolism*. Clarendon Press. Oxford University Press, New York. .

HEINEKE, D., BURGI, R., HEIKE, G., HOFERICHTER, P., PETER, U., FLÜGGE, U.I., & HELDT, H.W. (1991). Redox transfer across the inner chloroplast envelope membrane. *Plant Physiol* **95**: 1131-1137.

HELDT, W.H., WERDAN, K., MILOVANCEV, M., & GELLER, G. (1973). Alkalization of the chloroplast stroma caused by light-dependent proton flux into the thylakoid space. *Biochimistry Biophysics Acta* **314**(2): 224-241.

HIBBERD, D.J. & LEEDALE, G.F. (1971). A new algal class- The Eustigmatophyceae. *Taxon* **20**(4): 523-525.

KROTH, P.G., CHIOVITTI, A., GRUBER, A., MARTIN-JEZEQUEL, V., MOCK, T., PARKER, M.S., STANLEY, M.S., KAPLAN, A., CARON, L., WEBER, T., MAHESWARI, U., ARMBRUST, E.V., & BOWLER, C. (2008). A model for carbohydrate metabolism in the diatom Phaeodactylum tricornutum deduced from comparative whole genome analysis. *PLoS ONE* **3**(1): e1426.

LAZARO, J.J., SUTTON, C.W., NICHOLSON, S., & POWLS, R. (1986). Characterisation of two forms of phosphoribulokinase isolated from the green alga, Scenedesmus obliquus. *Eur J Biochem* **156**(2): 423-429.

LIAUD, M.F., BRANDT, U., SCHERZINGER, M., & CERFF, R. (1997). Evolutionary origin of cryptomonad microalgae: two novel chloroplast/cytosol-specific GAPDH genes as potential markers of ancestral endosymbiont and host cell components. *Journal of Molecular Evolution* **44 Suppl 1**: S28-37.

MABERLY, S.C., COURCELLE, C., GROBEN, R., & GONTERO, B. (2010). Phylogenetically-based variation in the regulation of the Calvin cycle enzymes, phosphoribulokinase and glyceraldehyde-3-phosphate dehydrogenase, in algae. *J Exp Bot* **61**(3): 735-745.

MEAD, R. & CURNOW, R.N. (1983). *Statistical methods in agriculture and experimental biology*. Chapman and Hall, London.

MICHELS, A.K., WEDEL, N., & KROTH, P.G. (2005). Diatom plastids possess a phosphoribulokinase with an altered regulation and no oxidative pentose phosphate pathway. *Plant Physiology* **137**(3): 911-920.

MOUSTAFA, A., BESZTERI, B., MAIER, U.G., BOWLER, C., VALENTIN, K., & BHATTACHARYA, D. (2009). Genomic Footprints of a Cryptic Plastid Endosymbiosis in Diatoms. *Science* **324**(5935): 1724-1726.

NICHOLSON, S., EASTERBY, J.S., & POWLS, R. (1987). Properties of a multimeric protein complex from chloroplasts possessing potential activities of NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase. *Eur J Biochem* **162**(2): 423-431.

O'BRIEN, M.J., EASTERBY, J.S., & POWLS, R. (1977). Glyceraldehyde-3-phosphate dehydrogenase of Scenedesmus obliquus. Effects of dithiothreitol and nucleotide on coenzyme specificity. *Biochim Biophys Acta* **481**(2): 348-358.

PETERSEN, J., TEICH, R., BECKER, B., CERFF, R., & BRINKMANN, H. (2006). The GapA/B gene duplication marks the origin of Streptophyta (charophytes and land plants). *Mol Biol Evol* **23**(6): 1109-1118.

POHLMEYER, K., PAAP, B.K., SOLL, J., & WEDEL, N. (1996). CP12: a small nuclear-encoded chloroplast protein provides novel insights into higher-plant GAPDH evolution. *Plant Mol Biol* **32**(5): 969-978.

PORTIS, A.R., JR. & HELDT, H.W. (1976). Light-dependent changes of the Mg^{2+} concentration in the stroma in relation to the Mg^{2+} dependency of CO_2 fixation in intact chloroplasts. *Biochim Biophys Acta* **449**(3): 434-436.

PUPILLO, P. & PICCARI, G.G. (1973). The effect of NADP on the subunit structure and activity of spinach chloroplast glyceraldehyde-3-phosphate dehydrogenase. *Arch Biochem Biophys* **154**(1): 324-331.

SCHURMANN, P. & JACQUOT, J.P. (2000). Plant Thioredoxin Systems Revisited. *Annu Rev Plant Physiol Plant Mol Biol* **51**: 371-400.

SPARLA, F., PUPILLO, P., & TROST, P. (2002). The C-terminal extension of glyceraldehyde-3-phosphate dehydrogenase subunit B acts as an autoinhibitory domain regulated by thioredoxins and nicotinamide adenine dinucleotide. *J Biol Chem* **277**(47): 44946-44952.

TAKISHITA, K., YAMAGUCHI, H., MARUYAMA, T., & INAGAKI, Y. (2009). A hypothesis for the evolution of nuclear-encoded, plastid-targeted glyceraldehyde-3-phosphate dehydrogenase genes in "chromalveolate" members. *PLoS One* **4**(3): e4737.

TAMOI, M., MIYAZAKI, T., FUKAMIZO, T., & SHIGEOKA, S. (2005). The Calvin cycle in cyanobacteria is regulated by CP12 via the NAD(H)/NADP(H) ratio under light/dark conditions. *Plant Journal* **42**(4): 504-513.

TROST, P., FERMANI, S., MARRI, L., ZAFFAGNINI, M., FALINI, G., SCAGLIARINI, S., PUPILLO, P., & SPARLA, F. (2006). Thioredoxin-dependent regulation of photosynthetic glyceraldehyde-3-phosphate dehydrogenase: autonomous vs. CP12-dependent mechanisms. *Photosynth Res* **89**(3): 1-13.

WEBER, T., GRUBER, A., & KROTH, P.G. (2009). The presence and localization of thioredoxins in diatoms, unicellular algae of secondary endosymbiotic origin. *Molecular Plant*: 1-10.

WERDAN, K., HELDT, H.W., & MILOVANCEV, M. (1975). The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on CO_2 fixation in the light and dark. *Biochim Biophys Acta* **396**(2): 276-292.

WILHELM, C., BUCHEL, C., FISAHN, J., GOSS, R., JAKOB, T., LAROCHE, J., LAVAUD, J., LOHR, M., RIEBESELL, U., STEHFEST, K., VALENTIN, K., & KROTH,

P.G. (2006). The regulation of carbon and nutrient assimilation in diatoms is significantly different from green algae. *Protist* **157**(2): 91-124.

WITTPOTH, C., KROTH, P.G., WEYRAUCH, K., KOWALLIK, K.V., & STROTMANN, H. (1998). Functional characterization of isolated plastids from two marine diatoms. *Planta* **206**(1): 79-85.

WOLOSIUK, R.A. & BUCHANAN, B.B. (1976). Studies on the regulation of chloroplast NADP-linked glyceraldehyde-3- phosphate dehydrogenase. *J Biol Chem* **251**(20): 6456-6461.

ZIMMERMANN, G., KELLY, G.J., & LATZKO, E. (1976). Efficient purification and molecular properties of spinach chloroplast fructose 1,6-bisphosphatase. *European Journal of Biochemistry* **70**(2): 361-367.

Figure legends

Fig. 1. pH-dependency of NADPH-dependent activity of GAPDH from three species. Three to four measurements at each pH value were performed with *A. formosa* (●),*C. reinhardtii* (○), and *P. ovalis* (●). Rates are presented as a percentage of maximal activities (nmol.min^{-1}.mg^{-1}) which were 682 for *A. formosa*, 491 for *C. reinhardtii* and 137 for *P. ovalis.* .

Fig. 2. Activation of GAPDH by dithiothreitol. The NADPH-dependent activity of GAPDH from *A. formosa* (●), *C. reinhardtii* (○) and *P. ovalis* (●), incubated with 20 mM DTT was followed at pH 7.7 as a function of time. The curves were fitted to experimental data using Equation 1 in Materials and Methods with A = 51 ± 3, c = 49 ± 3, λ = 0.5 ± 0.1 for *A. formosa*; A = 81 ± 4, c = 19 ± 2, λ = 0.12 ± 0.01 for *C. reinhardtii* and A = 58.25 ± 7.44, c = 42 ± 5, λ = 0.17 ± 0.06 for *P. ovalis. Q* is the rate of the reaction per mg of protein and the maximal activity (100%) of GAPDH was 594±31, 1180±70 and 160±20 nmol min^{-1} mg protein^{-1} for *A. formosa, C. reinhardtii* and *P. ovalis*, respectively.

Fig. 3. Comparison of NADPH-GAPDH activity from *P. ovalis* cells grown under dark or light, broken in the presence of different co-factors (0.1 mM) and measured without treatment or after incubation with either reduced or oxidised DTT. Means and one standard deviation are presented. Statistical comparisons (Student's t-test) are shown: comparisons within a block use the same letters to group activities that are not significantly different; comparisons above each column refer to the untreated versus treated samples; comparisons between the two panels, shown as a line in italics, refer to the difference between dark and light cells; *, $P<0.05$; **, $P<0.01$; ***, $P< 0.001$.

Fig. 4. Effect of NADPH on GAPDH from three species. Activity of samples incubated prior to assay with [NADPH] (circles), or for inhibited samples, after subsequent addition of 20 mM DTT (triangles) was measured: *P. ovalis* (A); *A. formosa* (B); and *C. reinhardtii* (C). Three to four measurements were performed for each treatment (mean and standard deviation are given).

Fig. 5. Inhibition of GAPDH by NADPH and pH dependency. Inhibition of GAPDH from *P. ovalis* (grey bar) and *A. formosa* (black bar) by 1 mM NADPH is expressed as a percentage of the activity of the reduced enzyme at each pH. Three to four measurements were performed for each treatment (mean and standard deviation are given).

Fig. 6. Effect of NADP on GAPDH from three species. Incubations of samples with NADP concentrations as indicated, prior to assay, were performed with: *P. ovalis* (A); *A. formosa* (B); and *C. reinhardtii* (C). Three to four measurements were performed for each treatment (mean and standard deviation are given).

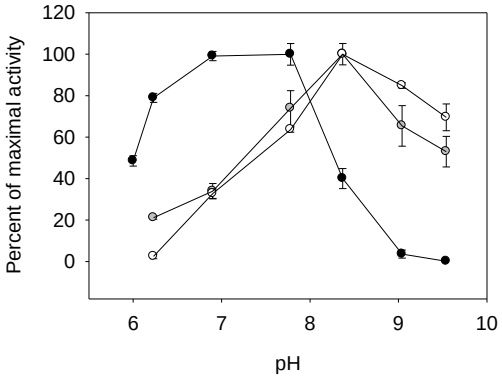


FIGURE 1

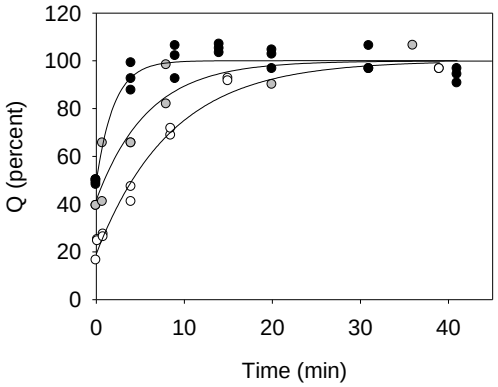
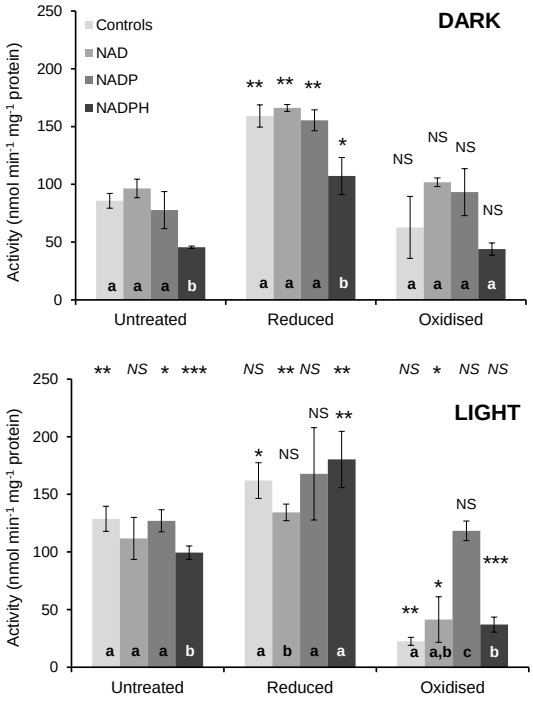


FIGURE 2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NADP
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Activity (nmol min-1 mg-1 protein)
250
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100
50
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** ** **
*
NS NS NS
NS
a a a b
a a a b
a a a a
Untreated
Reduced
Oxidised
LIGHT
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a a a b
a b a a
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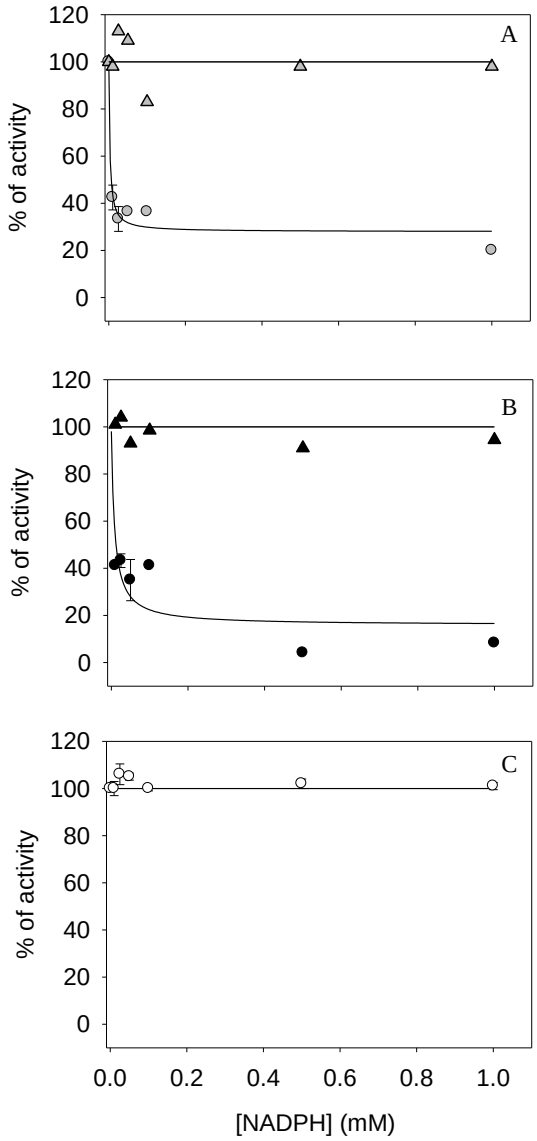
Untreated
Reduced
Oxidised

FIGURE 3


A
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1.0
[NADPH] (mM)

FIGURE 4

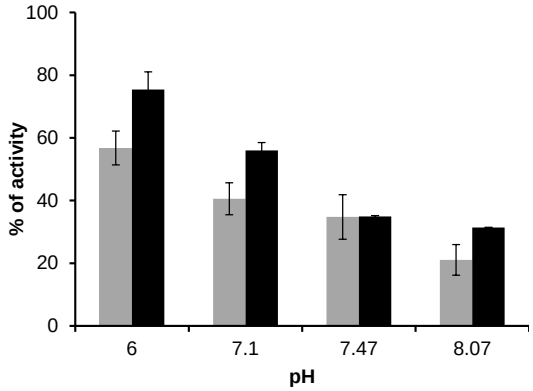

FIGURE 5

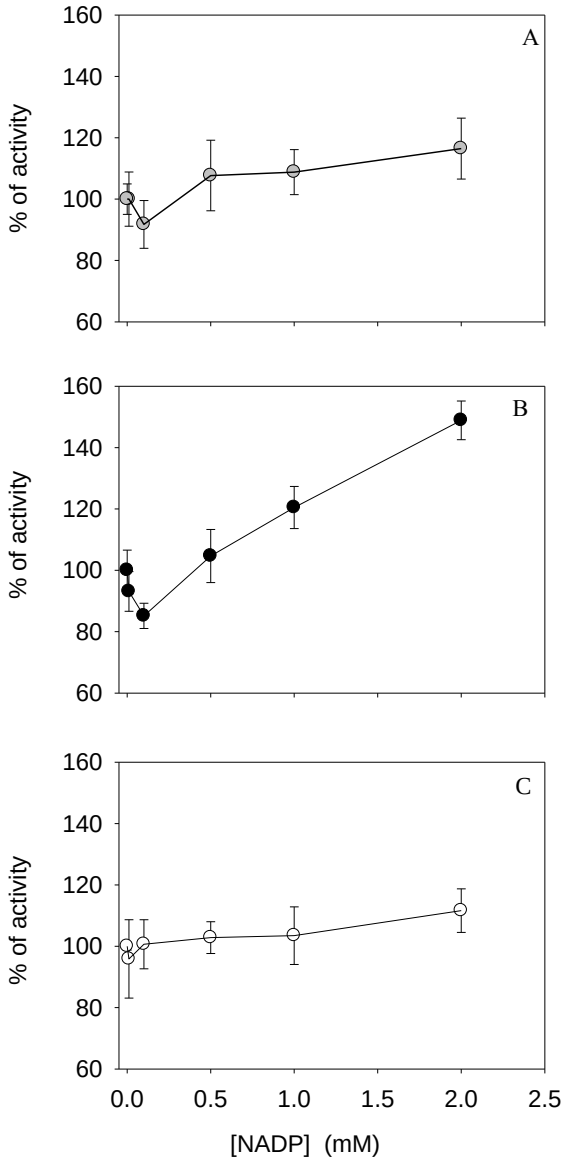

FIGURE 6