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Purification and properties of NADP-dependent non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase from the green alga *Chlamydomonas reinhardtii*

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NADP-dependent non-phosphorylating D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9), previously described in higher plants, has been now found to be present in eukaryotic green algae, but in neither cyanobacteria nor non-photosynthetic microorganisms. The enzyme from the unicellular green alga *Chlamydomonas reinhardtii*, strain 6145c, has been purified to apparent electrophoretic homogeneity. The non-phosphorylating enzyme was effectively separated from the NADP-dependent phosphorylating D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) by dye-ligand chromatography on Reactive Red-120 agarose. The purified enzyme exhibited an optimum pH in the 8.5–9.0 range and a specific activity of approx. 8 μmol · (mg protein)$^{-1}$ · min^{-1}. The native protein was characterized as a homotetramer with a molecular weight of 190 000, a Stokes radius of 5.2 nm, and an isoelectric point of 6.9. From kinetic studies, K_m-values of 9.8 and 51 μM were calculated for NADP and D-glyceraldehyde 3-phosphate, respectively, an absolute specificity for both substrates being observed. L-Glyceraldehyde 3-phosphate was a potent non-competitive inhibitor (K_i, 48 μM). The reaction products NADPH and D-3-phosphoglycerate inhibited enzyme activity in a competitive manner with respect to NADP (K_i, 78 μM) and D-glyceraldehyde 3-phosphate (K_i, 1.2 mM), respectively. Thermal inactivation occurred above 45 °C and was effectively prevented by either substrate. The presence of essential vicinal thiol groups is suggested by the inactivation produced by diamide, with D-glyceraldehyde 3-phosphate, but not NADP, behaving as a protective agent. The enzyme's possible physiological role in photosynthetic metabolism is discussed briefly.

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Abbreviations: diamide, azodicarboxylic acid bis(dimethylamide); glyceraldehyde-3-phosphate:NADP oxidoreductase, NADP-dependent non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; Mops, 4-morpholinepropanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine.

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Introduction

From the early reports of Arnon and co-workers [1,2] it seems clear that three different glyceraldehyde-3-phosphate dehydrogenases exist in photosynthetic tissues of higher plants: (i) a cytosolic NAD-dependent phosphorylating dehydrogenase (EC 1.2.1.12), which catalyzes the reversible, P_i-dependent oxidation of glyceraldehyde 3-phosphate

to 1,3-bisphosphoglycerate [3–5]:

$$\text{NAD} + \text{glyceraldehyde 3-phosphate} + P_i \rightleftharpoons 1,3\text{-bisphosphoglycerate} + \text{NADH} + H^+$$

(ii) a chloroplastic NADP-dependent phosphorylating dehydrogenase (EC 1.2.1.13), which catalyzes the same reversible reaction and is involved in photosynthetic CO_2 assimilation through the Calvin cycle [4–7]; and (iii) a cytosolic NADP-dependent non-phosphorylating dehydrogenase (EC 1.2.1.9), namely glyceraldehyde 3-phosphate : NADP oxidoreductase, which catalyzes the irreversible, P_i-independent oxidation of glyceraldehyde 3-phosphate to 3-phosphoglycerate, according to the following reaction:

$$\text{NADP} + \text{glyceraldehyde 3-phosphate} + H_2O \rightarrow \text{3-phosphoglycerate} + \text{NADPH} + 2\,H^+$$

The two phosphorylating enzymes have been extensively studied and both their physicochemical and kinetic properties are well known [7–11]. On the other hand, reports on the non-phosphorylating dehydrogenase are scarce, the enzyme having been described and characterized only in higher plant tissues [7,9,10]. Gibbs and co-workers have proposed the involvement of this enzyme in a shuttle system for the export of photosynthetically generated reducing equivalents (NADPH) from chloroplast to cytoplasm [12,13].

Although the NADP-dependent phosphorylating glyceraldehyde-3-phosphate dehydrogenase has been described and characterized in microalgae [5,14,15], to the best of our knowledge no data are available concerning the NADP-dependent non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase from these microorganisms. In this paper we report on the occurrence of this enzyme in eukaryotic algae and its purification and characterization from the green alga *Chlamydomonas reinhardtii*. Its possible metabolic role in photosynthesis is also discussed.

Materials and Methods

Organisms and culture conditions

The green algae *Chlamydomonas reinhardtii* strain 6145c (from Dr. R. Sager, Hunter College, New York), *Monoraphidium braunii* (formerly *Ankistrodesmus braunii*) strain 202-7c and *Chlorella fusca* strain 211-15 (from Göttingen University's Culture Collection) were grown at 25 °C in liquid media as previously described [16,17]. The cyanobacteria (blue-green algae) *Anacystis nidulans* (reclassified as *Synechococcus leopoliensis*) strain 1402-1 (from Göttingen University's Culture Collection), *Synechocystis* sp. PCC 6803 (from the Pasteur Institute Culture Collection) and *Calothrix* sp. (formerly *Tolypothrix tenuis*) PCC 7101 (from the Pasteur Institute Culture Collection) were grown on a modified BG-11 synthetic medium [18]. *Anabaena* sp. ATCC 33047 (from American Type Culture Collection) was grown as previously described [19]. All strains were cultured photoautotrophically under continuous fluorescent illumination (25 $W \cdot m^{-2}$) using a gas stream of 5% (v/v) CO_2 in air. Spinach (*Spinacia oleracea* L.) leaves were obtained from a local greenhouse.

Algal cells were harvested at the late exponential growth phase by centrifugation at 12000 × *g* for 10 min. For enzyme purification, *C. reinhardtii* cells were harvested under the same conditions but using a Szent-Györgyi-Blum continuous flow system (Ivan Sorvall, Inc. U.S.A.). The cells were washed twice in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 10 mM 2-mercaptoethanol and then stored frozen as a paste at −20 °C before use. The yield of *C. reinhardtii* cells was 3–4 g (wet weight) per liter of culture medium.

Preparation of cell-free extracts

Immediately prior to use algal cells were resuspended in the above wash medium at a ratio of about 2 ml buffer per g (wet weight). The cells were then disrupted in the cold with a Branson model B-12 sonifier (120 s, 80 W), and cell-free extracts were obtained by centrifugation at 40000 × *g* for 20 min. Freshly harvested leaves of spinach were homogenized in the cold using the same buffer (1 ml per g) in a Waring Blendor at full speed for four 20-s intervals and were centrifuged as above.

Analytical procedures

Enzyme assays. Glyceraldehyde-3-phosphate : NADP oxidoreductase activity was determined spectrophotometrically at 30 °C by monitoring

NADPH generation at 340 nm in a Pye Unicam SP8-150 recording spectrophotometer. The standard assay medium contained 50 mM Tricine-NaOH buffer (pH 8.5), 0.4 mM NADP, 1 mM D-glyceraldehyde 3-phosphate and an adequate quantity of enzyme in a total volume of 1 ml. NADP-dependent phosphorylating glyceraldehyde-3-phosphate dehydrogenase was assayed using the same reaction medium but supplemented with 10 mM Na_2HAsO_4. One unit of enzyme is defined as the amount which catalyzes the formation of 1 μmol NADPH per min under the specified conditions. The reverse reaction was assayed by following the 1,3-bisphosphoglycerate-dependent oxidation of NADPH according to Ferri et al. [20]. For determination of optimal pH, Tricine buffer was replaced by an equimolar mixture of Mops/Tricine/glycine (total concentration, 60 mM), and the pH value in the range 6–10 was adjusted by addition of NaOH.

Protein measurements. Total protein was determined either by a modification of the method of Lowry et al. [21] or according to Bradford [22], and bovine serum albumin was used as a standard.

Gel electrophoresis. Non-denaturing polyacrylamide gel electrophoresis was carried out as described by Jovin et al. [23] on tube gels (5 × 90 mm) of 7.5% (w/v) acrylamide, with a stacking gel of 3.5% (w/v) acrylamide. Proteins were stained with 1% (w/v) Coomassie brilliant blue in 7% (v/v) acetic acid for 4 h at room temperature. The glyceraldehyde-3-phosphate : NADP oxidoreductase activity was located in the gels by the appearance of NADPH fluorescence under ultraviolet light (Sylvania BLB 15 W). The gels were soaked for 20 min in the standard reaction medium and the enzyme was located as a yellow-green fluorescent band by placing the gels on a black background under lateral illumination. For densitometric scans, gels processed for enzyme activity were placed in cylindrical glass cells with a light path of 0.5 cm, and absorbance at 340 nm was recorded at 0.4 mm · s^{-1} using a Pye-Unicam SP8-100 spectrophotometer equipped with a densitometer. Denaturing electrophoresis in the presence of sodium dodecylsulfate was performed according to Laemmli [24] using 7.5% (w/v) acrylamide slab gels.

Molecular weight determination. The molecular weight of native *C. reinhardtii* glyceraldehyde-3-phosphate : NADP oxidoreductase was determined by using a 1.5 × 40 cm column of Sephacryl S-300 Superfine calibrated with protein markers of known molecular weight, and 1-ml fractions were collected and checked for both absorbance at 280 nm and enzyme activity (see legend of Fig. 2A). Subunit analysis was performed by electrophoresis in the presence of sodium dodecyl sulfate [24] using the molecular weight markers indicated in Fig. 2B.

Isoelectric point determination. The isoelectric point of native glyceraldehyde-3-phosphate : NADP oxidoreductase purified from *C. reinhardtii* was estimated by analytical chromatofocusing (see legend of Fig. 3) using a 1 × 18 cm Polybuffer Exchanger 94 column according to the specifications of the manufacturer (Chromatofocusing; Pharmacia Fine Chemicals, Uppsala, Sweden, 1980).

Purification of glyceraldehyde-3-phosphate : NADP oxidoreductase from C. reinhardtii

Unless otherwise stated all operations were performed at 0–4° C. Centrifugations were carried out at 40000 × *g* for 20 min.

(i) Extraction and ammonium sulfate fractionation. C. reinhardtii cells (40 g, wet weight) were sonicated as described above, the supernatant fraction obtained by centrifugation (about 100 ml) being defined as the crude extract. This cell-free, green-colored preparation was brought to 40% (w/v) saturation with solid ammonium sulfate, and the suspension was gently stirred for 20 min and then centrifuged. The yellowish supernatant fluid was brought to 80% (w/v) saturation by further addition of solid ammonium sulfate, and the precipitate was collected by centrifugation and dissolved in 50 ml of 25 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM EDTA and 10 mM 2-mercaptoethanol (standard buffer). The protein solution was dialyzed twice against 2 l of the same buffer and eventually centrifuged, the resulting supernatant fluid being used for the next step.

(ii) DEAE-cellulose ion-exchange chromatography. The dialyzed, clarified protein solution was applied to a Whatman DE-52 anion-exchange column (3 × 8 cm) equilibrated with standard buffer.

The column was then washed with 4 bed volumes of the same buffer at a flow rate of 30 $ml \cdot h^{-1}$. Both glyceraldehyde-3-phosphate : NADP oxidoreductase and NADP-dependent phosphorylating glyceraldehyde-3-phosphate dehydrogenase eluted together as a broad peak slightly retarded with respect to the buffer front during washing, conditions under which most of the yellow material remained tightly bound to the DEAE-cellulose bed. The active fractions were collected, and poly(ethyleneglycol)-4000 was then added to the pool up to a final concentration of 5% (w/v).

(iii) Reactive Red-120 agarose dye-ligand chromatography. The protein solution was next applied to a Reactive Red-120 agarose column (1.5 × 17 cm) equilibrated with standard buffer containing 25% (v/v) glycerol. The column was washed with 3 bed volumes of the equilibration buffer and subsequently with 5 bed volumes of the same buffer supplemented with 0.3 M NaCl. The flow rate was 20 $ml \cdot h^{-1}$. Whereas glyceraldehyde-3-phosphate : NADP oxidoreductase passed immediately through the agarose bed, the NADP-dependent phosphorylating glyceraldehyde-3-phosphate dehydrogenase remained tightly bound to the column, being subsequently eluted with a linear gradient of NaCl (0.3–1.0 M; total volume 200 ml) in equilibration buffer at a flow rate of 10 $ml \cdot h^{-1}$. The glyceraldehyde-3-phosphate : NADP oxidoreductase preparation was concentrated and washed with 25 mM Tris-HCl buffer (pH 9.0) containing 0.1 mM EDTA and 10 mM 2-mercaptoethanol (starting buffer) by ultrafiltration on a Diaflo PM-10 Amicon membrane.

(iv) Chromatofocusing. Column chromatofocusing in the pH range 9.0–4.0 was performed on a Polybuffer Exchanger 94 column (1 × 18 cm) equilibrated with starting buffer. After application of the concentrated glyceraldehyde-3-phosphate : NADP oxidoreductase solution (about 10 ml), the column was washed with 5 ml of starting buffer. The enzyme was eluted at a flow rate of 12 $ml \cdot h^{-1}$ by washing the column with 10 bed volumes of a 10-fold diluted mixture of Polybuffer 96/Polybuffer 74 (30/70 (v/v)) adjusted to pH 4.0 with acetic acid. The pooled active fractions were concentrated and equilibrated in standard buffer supplemented with 0.1 M NaCl by ultrafiltration as described above.

(v) Sephacryl S-300 gel filtration. The concentrated glyceraldehyde-3-phosphate : NADP oxidoreductase solution (2 ml) was applied to a column of Sephacryl S-300 (1.6 × 40 cm) equilibrated with standard buffer supplemented with 0.1 M NaCl, and eluted at a flow rate of 20 $ml \cdot h^{-1}$. The active fractions were pooled, concentrated and equilibrated in standard buffer by ultrafiltration and stored at 4 °C, under which conditions enzyme activity remained essentially unchanged for at least 1 month. This protein solution was used as the final enzyme preparation.

Chemicals

NADP, NAD, NADPH, NADH, D-glyceraldehyde 3-phosphate, DL-glyceraldehyde 3-phosphate, D-3-phosphoglycerate, yeast 3-phosphoglycerate kinase, Reactive Red-120, Reactive Red-120 agarose, Tris, Tricine, Mops, glycine, azodicarboxylic acid bis(dimethylamide), bovine serum albumin, EDTA, dithioerythritol, erythrose 4-phosphate, poly(ethyleneglycol)-4000 and hydroxypyruvic acid phosphate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Polybuffer Exchanger 94, Polybuffer 74, Polybuffer 96, Sephacryl S-300 Superfine and protein standards for gel filtration and electrophoresis were from Pharmacia Fine Chemicals (Uppsala, Sweden); acrylamide and methylenebisacrylamide were from BDH (Poole, U.K.); DEAE-cellulose DE-52 was from Whatman Inc. (Maidstone, U.K.). All other reagents were of analytical grade and acquired from Merck (Darmstadt, F.R.G.).

NADPH and NADP concentrations were determined from the molar absorption coefficient of $6.22 \cdot 10^3$ $cm^{-1} \cdot M^{-1}$ at 340 nm and $18.0 \cdot 10^3$ $cm^{-1} \cdot M^{-1}$ at 260 nm, respectively. D-Glyceraldehyde 3-phosphate and DL-glyceraldehyde 3-phosphate concentrations were determined by enzymatic assay as described by the manufacturer (Sigma Chemical Co.).

Results and Discussion

Distribution of glyceraldehyde-3-phosphate : NADP oxidoreductase activity in photosynthetic organisms

Since no previous studies have been reported on the presence of glyceraldehyde-3-phosphate :

NADP oxidoreductase activity in eukaryotic algae, we have checked for the occurrence of this enzyme in several representative green algae (Table I). Moreover, as information on the distribution of this enzyme is insufficient and somewhat confusing [25], we have examined several strains of cyanobacteria as well as prokaryotic and eukaryotic non-photosynthetic microorganisms, namely, *Azotobacter chroococcum* and Baker's yeast (*Saccharomyces cerevisiae*). It was also of interest for comparative purposes to assess the NADP-dependent phosphorylating glyceraldehyde-3-phosphate dehydrogenase activity level in the corresponding cell-free extracts.

Whereas NADP-dependent phosphorylating glyceraldehyde-3-phosphate dehydrogenase was present, showing specific activity values of 25–40 m units per mg protein in all the photosynthetic organisms examined, glyceraldehyde-3-phosphate : NADP oxidoreductase activity was found (specific activity 5–7 m units per mg protein) only in eukaryotes, both green algae and a higher plant, but was not detected in cyanobacteria, a group of photosynthetic prokaryotes which perform oxygenic photosynthesis (Table I). Attempts to reveal either NADP-dependent enzyme in a non-photosynthetic bacterium and in Baker's yeast were unsuccessful (data not shown).

TABLE I

DISTRIBUTION OF NADP-DEPENDENT NON-PHOSPHORYLATING AND PHOSPHORYLATING GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE ACTIVITY IN SEVERAL PHOTOSYNTHETIC ORGANISMS

The preparation and assay of cell-free extracts were as described under Materials and Methods. Glyceraldehyde-3-phosphate: NADP oxidoreductase was further characterized in crude extracts by the inhibition (about 95%) caused by 0.5 mM L-glyceraldehyde 3-phosphate (added as DL-glyceraldehyde 3-phosphate), which had no effect on the phosphorylating activity. n.d., not detected.

Organism	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase activity (mU · (mg protein)$^{-1}$)	
	non-phosphorylating	phosphorylating
Spinacia oleracea	6	32
Chlamydomonas reinhardtii	5	28
Monoraphidium braunii	6	35
Chlorella fusca	7	27
Anacystis nidulans	n.d.	40
Anabaena sp. ATCC 33047	n.d.	25
Synechocystis sp. PCC 6803	n.d.	38
Calothrix sp. PCC 7101	n.d.	32

Purification of glyceraldehyde-3-phosphate: NADP oxidoreductase from C. reinhardtii

Glyceraldehyde-3-phosphate : NADP oxidoreductase was purified about 1600-fold from the green alga *C. reinhardtii*. The devised purification protocol, summarized in Materials and Methods and Table II, yielded electrophoretically pure enzyme with a specific activity of approx. 8 μmol · (mg protein)$^{-1}$ · min^{-1} and a recovery of 34%. The specific activity value obtained for the purified algal oxidoreductase is very similar to that reported for the higher plant enzyme [7].

It must be noted that although attempts by different purification techniques failed to resolve the activities of NADP-dependent phosphorylating glyceraldehyde-3-phosphate dehydrogenase and glyceraldehyde-3-phosphate : NADP oxidoreductase, this objective was effectively achieved using dye-ligand chromatography. The striking differential adsorption on Reactive Red-120 agarose exhibited by the two enzymes – despite the similarity in the reactions they catalyze – is a notable feature of the purification protocol. This chromatographic behavior of the enzymes is in accord with their kinetic properties studied in free solution in the presence of Reactive Red-120, i.e., whereas the activity of glyceraldehyde-3-phosphate : NADP oxidoreductase – which passed unretarded through the column – was unaffected by the dye up to 2 mM, Reactive Red was a powerful competitive inhibitor (K_i, 0.15 μM) with respect to NADP of the NADP-dependent phosphorylating glyceraldehyde-3-phosphate dehydrogenase – which remained tightly adsorbed to the bed. The presence of 5% poly(ethyleneglycol)-4000 and 25% glycerol during this purification step stabilized the NADP-dependent phosphorylating glyceraldehyde-3-phosphate dehydrogenase and allowed verification of the complete resolution of both enzymes.

Glyceraldehyde-3-phosphate : NADP oxidoreductase was eventually purified to apparent elec-

TABLE II

PURIFICATION OF GLYCERALDEHYDE-3-PHOSPHATE: NADP OXIDOREDUCTASE FROM *C. REINHARDTII*

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units·(mg protein)$^{-1}$)	Purification factor (fold)	Yield (%)
Crude extract	3280	16.4	0.005	1	100
Ammonium sulfate (40–80% fractionation)	457	10.6	0.023	5	65
DEAE-cellulose eluate	76	9.5	0.125	25	58
Reactive Red-120 agarose eluate	14	8.3	0.593	119	51
Chromatofocusing (pH 9.0–4.0) eluate	1.5	6.2	4.13	826	38
Sephacryl S-300 eluate	0.7	5.5	7.86	1571	34

trophoretic homogeneity by column chromatofocusing and Sephacryl S-300 gel filtration. Chromatofocusing was employed by exploiting the almost neutral isoelectric point of the enzyme (see Fig. 3, below), which subsequently eluted from the Sephacryl S-300 column as a symmetrical peak with constant specific activity. Homogeneity of the resulting preparation was checked by non-denaturing electrophoresis, a single protein band on 7.5% polyacrylamide gels with glyceraldehyde-3-phosphate : NADP oxidoreductase activity being observed (Fig. 1). As discussed below, the purified enzyme preparation was also homogeneous when subjected to electrophoresis in the presence of sodium dodecyl sulfate.

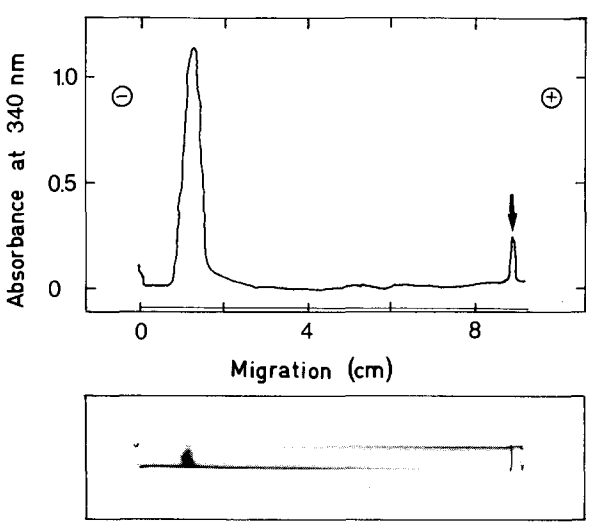

Fig. 1. Non-denaturing polyacrylamide gel electrophoresis of purified *C. reinhardtii* glyceraldehyde-3-phosphate : NADP oxidoreductase. The enzyme (25 μg protein) was run in 7.5% polyacrylamide tube gels. The upper figure corresponds to a densitometric scan at 340 nm of a gel stained for enzyme activity. The arrow indicates the position of the tracking dye. The photograph below shows a gel stained for protein.

Physical properties

Molecular weight determination of native glyceraldehyde-3-phosphate : NADP oxidoreductase from *C. reinhardtii* was performed by gel filtration on a Sephacryl S-300 column and yielded a value of 190 000 (Fig. 2A). Gel electrophoresis of the purified enzyme under denaturing conditions is shown in Fig. 2B, the single stained protein band indicating a molecular weight of about 50 000 for the oxidoreductase subunit. Thus, a homotetrameric structure seems to be predominant under the conditions employed, in agreement with the data reported for glyceraldehyde-3-phosphate : NADP oxidoreductases from higher plants [7,9]. Estimation by Sephacryl S-300 gel filtration of the Stokes radius of the algal oxidoreductase gave a value of 5.2 nm. The absorption spectrum of purified algal oxidoreductase showed no peak in the visible range, a typical protein absorption band being observed at 274 nm.

Fig. 3 shows the elution profile of purified *C. reinhardtii* glyceraldehyde-3-phosphate : NADP oxidoreductase subjected to analytical chromatofocusing. A constant isoelectric point of 6.9 was estimated using different enzyme preparations. This near-neutral isoelectric point can account for the poor adsorption of the enzyme on DEAE-cellulose. The only previous determination

of this parameter corresponds to the oxidoreductase from *Beta vulgaris* [7], with an estimated p*I* value of 6.0.

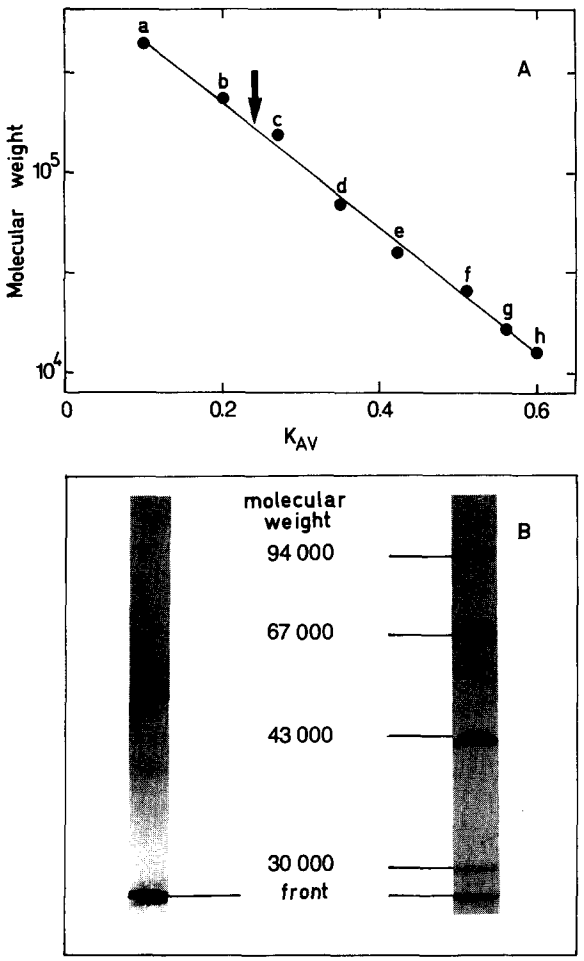

Fig. 2. Determination of the molecular weight of native (A) and denatured (B) glyceraldehyde-3-phosphate : NADP oxidoreductase from *C. reinhardtii*. (A) The enzyme (about 0.5 mg in 0.5 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM EDTA, 10 mM 2-mercaptoethanol and 0.1 M NaCl) was applied to a column (1.5×40 cm) of Sephacryl S-300 equilibrated with the same buffer. Elution was carried out at a flow rate of 15 ml·h^{-1} at 4° C. The column was calibrated with the following molecular weight protein markers: ferritin (a), 440000; catalase (b), 232000; aldolase (c), 158000; bovin serum albumin (d), 67000; ovalbumin (e), 43000; chymotrypsinogen A (f), 25000; myoglobin (g), 16900; and RNAase A (h), 13700. The arrow indicates the K_{AV}-value obtained for glyceraldehyde-3-phosphate : NADP oxidoreductase. (B) Purified enzyme (20 μg) was applied to a polyacrylamide slab gel in the presence of sodium dodecyl sulfate. Electrophoresis was conducted overnight at 4° C by applying a constant current of 6 mA. On completion, the gel was stained with Coomassie brilliant blue to locate proteins.

Kinetic properties

Purified *C. reinhardtii* glyceraldehyde-3-phosphate : NADP oxidoreductase was absolutely specific for both NADP and the D-form of glyceraldehyde 3-phosphate. From double-reciprocal plots of reaction rates under standard assay conditions in the presence of saturating concentrations of one substrate, apparent K_m-values for NADP and D-glyceraldehyde 3-phosphate were estimated to be 9.8 and 51 μM, respectively. L-Glyceraldehyde 3-phosphate was a potent noncompetitive inhibitor of the algal enzyme with respect to the D-form (K_i, 48 μM). These results are in agreement with substrate specificities and K_m values reported for the higher plant enzyme [2,9,12,26]. The optimum pH value for the algal oxidoreductase was in the 8.5–9.0 range. Since values about 7 have been claimed for the cytoplasmic pH in eukaryotic algae [27], apparent K_m values for both substrates were also determined at this pH and found to be 11 and 32 μM for NADP and D-glyceraldehyde 3-phosphate, respectively. When 1 mM D-3-phosphoglycerate (or 1,3-bisphosphoglycerate) and 0.5 mM NADPH (or NADH) were incubated with the purified oxidoreductase, no NADPH (or NADH) oxidation was observed in the pH range 6–10 under otherwise standard conditions, as might be expected from the apparent equilibrium constant (K'_{eq} at pH 7, $2 \cdot 10^7$) calculated from the midpoint redox potentials of the pairs involved (cf. Ref. 30).

Preincubation of purified oxidoreductase for 5 min at temperatures in the 0–40° C range did not irreversibly affect enzyme activity. Thermal inactivation did, however, occur above 45° C and resulted in total activity loss at 55° C. Interestingly, this inactivation was effectively prevented by both NADP and D-glyceraldehyde 3-phosphate. Studies on the effect of assay temperature on enzyme activity revealed an optimal value of about 55° C, an activation energy of 38.5 kJ·mol^{-1} being calculated from the corresponding linear Arrhenius plot (data not shown).

Table III shows that algal glyceraldehyde-3-phosphate : NADP oxidoreductase was markedly inhibited by the sulfhydryl-modifying reagents iodoacetamide and diamide. Whereas D-glyceraldehyde 3-phosphate behaved as a protec-

TABLE III

INHIBITION OF *C. REINHARDTII* GLYCERALDEHYDE-3-PHOSPHATE : NADP OXIDOREDUCTASE BY SULFHYDRYL-MODIFYING REAGENTS

The purified enzyme (about 0.5 mg protein ml^{-1}) was incubated at 25 °C in 50 mM Mops-NaOH (pH 7.2) with the indicated additions. After 30 min, aliquots were withdrawn, diluted 100-fold in the assay medium and assayed for activity. Controls were similarly treated except that sulfhydryl reagents were absent. The presence of the sulfhydryl reagents (100-fold diluted) in the assay medium had no effect on enzyme activity.

Additions	Remaining activity (% of control)
None	100
Iodoacetamide (0.4 mM)	56
Iodoacetamide (0.4 mM), D-glyceraldehyde 3-phosphate (1 mM)	100
Iodoacetamide (0.4 mM), NADP (1 mM)	46
Diamide (0.4 mM)	50
Diamide (0.4 mM), D-glyceraldehyde 3-phosphate (1 mM)	101
Diamide (0.4 mM), NADP (1 mM)	48
Diamide (0.4 mM), then dithioerythritol [a] (10 mM)	110

[a] After the 30-min incubation with diamide, excess reagent was removed from the medium according to Penefsky [32]. The desalted enzyme was then incubated with 10 mM dithioerythritol for 10 min before assay.

tive agent, no protection by NADP was observed. Dithioerythritol completely reactivated the diamide-treated enzyme, thus showing that this inactivation occurs through oxidation of vicinal dithiols [28]. These results suggest the participation of essential sulfhydryl groups in the catalytic activity of glyceraldehyde-3-phosphate : NADP oxidoreductase.

The effect of various coenzymes and metabolites which might affect the activity of *C. reinhardtii* glyceraldehyde-3-phosphate : NADP oxidoreductase in vivo has also been investigated (Table IV). Both reaction products, NADPH and D-3-phosphoglycerate, were inhibitors. NADH, but not NAD, also inhibited enzyme activity, although to a much lesser degree than NADPH. Orthophosphate (and arsenate) were slightly inhibitory. On

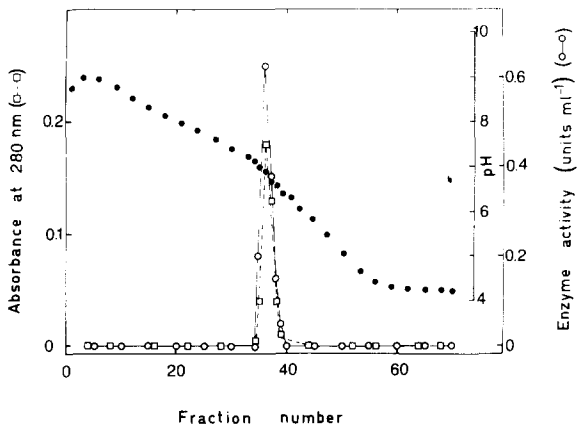

Fig. 3. Analytical chromatofocusing of purified *C. reinhardtii* glyceraldehyde-3-phosphate : NADP oxidoreductase. A sample containing 0.6 mg of native protein was applied to a Polybuffer Exchanger 94 column (1×18 cm) equilibrated with 25 mM Tris-HCl buffer (pH 9.0) containing 0.1 mM EDTA and 10 mM 2-mercaptoethanol. The enzyme was eluted by using a pH gradient (●) generated by 10 bed volumes of a 10-fold diluted mixture of Polybuffer 96/Polybuffer 74 (30/70 (v/v)) adjusted with acetic acid to pH 4.0. Fractions of 1.2 ml were collected. Absorbance at 280 nm (□) and enzyme activity (○) were measured for each fraction.

the other hand, AMP, ADP, and ATP were without effect. Phosphohydroxypyruvate, an intermediate in the conversion of 3-phosphoglycerate to serine [29], and also erythrose 4-phosphate, a metabolite

TABLE IV

EFFECT OF VARIOUS COENZYMES AND METABOLITES ON THE ACTIVITY OF GLYCERALDEHYDE-3-PHOSPHATE : NADP OXIDOREDUCTASE FROM *C. REINHARDTII*.

Activity was measured in the presence of the indicated compound as described in the text.

Compound	Relative activity (% of control)
None	100
NADPH (0.4 mM)	48
NADH (0.4 mM)	87
NAD (2 mM)	100
D-3-Phosphoglycerate (2 mM)	72
Orthophosphate (10 mM)	80
AMP (2 mM)	99
ADP (2 mM)	100
ATP (2 mM)	100
Dihydroxyacetone phosphate (2 mM)	98
Erythrose 4-phosphate (2 mM)	41
Phosphohydroxypyruvate (0.1 mM)	30

of the NADPH-generating oxidative pentose phosphate pathway, behaved as efficient inhibitors, whereas dihydroxyacetone phosphate was ineffective. Other compounds tested and found to have little or no influence (at 2 mM concentration) on enzyme activity were phospho*enol*pyruvate, D-glucose 6-phosphate, D-fructose 6-phosphate, and D-fructose 1,6-bisphosphate. These results agree in general with those reported by Kelly and Gibbs [10] for the higher plant enzyme.

More detailed kinetic analysis of the inhibition by products (see Table IV) showed NADPH to behave as a competitive inhibitor (K_1, 78 μM) with respect to NADP (Fig. 4A), and D-3-phosphoglycerate (K_i, 1.2 mM) with respect to D-glyceraldehyde 3-phosphate (Fig. 4B). The possible physiological relevance of these inhibitions remains to be established.

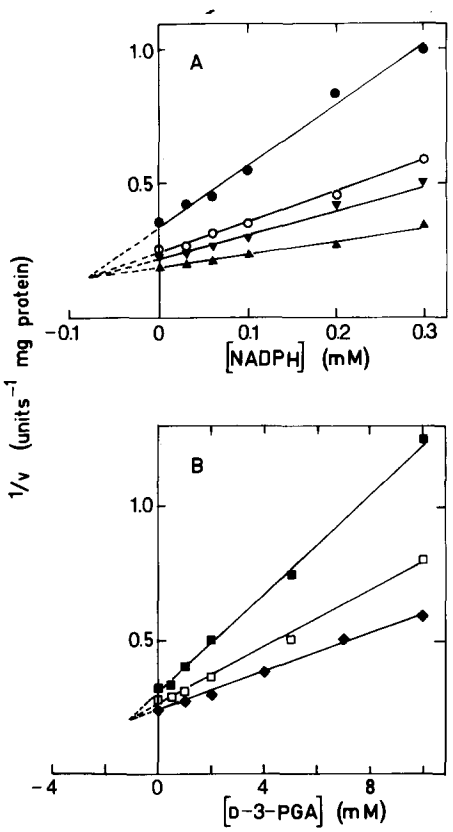

Fig. 4. Product inhibition of *C. reinhardtii* glyceraldehyde-3-phosphate:NADP oxidoreductase (Dixon plots). Enzyme activity was assayed as described under Materials and Methods except that the following changes were made: (A) NADPH was added as indicated; NADP concentrations were 20 (▲), 40 (▼), 70 (○), and 200 (●) μM. (B) D-3-Phosphoglycerate (D-3-PGA) was present in the assay medium as indicated; D-glyceraldehyde 3-phosphate concentrations were 100 (◆), 200 (□), and 500 (■) μM.

These results are the first to be reported on the occurrence and characterization of NADP-dependent non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase in green algae. Since this enzyme seems to be restricted to eukaryotic photosynthetic organisms and to be located in the cytoplasm rather than in the chloroplast [2,10], it may be involved in the indirect shuttling not only of reducing equivalents [12,13], but also of protons (cf. Ref. 30) and 3-phosphoglycerate, from chloroplast to cytoplasm, and consequently in the maintenance of a high level of cytosolic NADPH and in the establishment of a ΔpH of about 0.8 units (cf. Ref. 31) between the chloroplast stroma and the cytosol.

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