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Characterization of native and recombinant A_4 glyceraldehyde 3-phosphate dehydrogenase

Kinetic evidence for conformation changes upon association with the small protein CP12

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A_4 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purified from the green alga *Chlamydomonas reinhardtii* and was also overexpressed in *Escherichia coli*. Both purified A_4 tetramers of recombinant and native GAPDH were characterized for the first time. The pH optimum for both native and recombinant enzymes was close to 7.8. The p*K*s of the residues involved in catalysis indicate that a cysteine and a histidine may take part in catalysis by chloroplast GAPDH, as is the case for glycolytic GAPDH. Native and recombinant GAPDH show Michaelis–Menten kinetics with respect to their cofactors, NADH and NADPH, with greater specificity for NADPH. The kinetic parameters are similar to those of the heterotetrameric A_2B_2 spinach chloroplast GAPDH. Native *C. reinhardtii* and recombinant GAPDHs exhibit a cooperative behavior towards the substrate 1,3-bisphosphoglycerate (BPGA). This positive cooperativity is specific to the *C. reinhardtii* enzyme, as higher plant A_2B_2 GAPDHs show Michaelis–Menten kinetics. Native GAPDH has twofold lower catalytic constant and $K_{0.5}$ for BPGA than recombinant GAPDH. Mass spectrometry analysis of native GAPDH shows that it is a complex of GAPDH and the small protein CP12. *In vitro* reconstitution assays indicate that the kinetic differences are the result conformation changes of GAPDH upon association with CP12.

Keywords: GAPDH; CP12; overexpression; purification; kinetics.

The enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) exists as two main forms in higher plants and algae. The cytosolic form is involved in glycolysis, while the chloroplast form is involved in the Benson–Calvin cycle. In this pathway, which is responsible for CO_2 assimilation, the chloroplast enzyme catalyzes the reversible reduction and dephosphorylation of 1,3-bisphosphoglycerate (BPGA) to glyceraldehyde 3-phosphate using NADPH generated by photosystem I in the light.

The GAPDH isolated from chloroplasts (EC 1.2.1.13) has dual specificity, and can use either NAD(H) or NADP(H). It has been suggested that GAPDH in higher plants exists either as a heterotetramer of two A subunits (36 kDa) and two B subunits (39 kDa) (A_2B_2), or as a homotetramer of four A subunits (A_4) [1]. A 600 kDa aggregated form (A_8B_8) has also been isolated from higher plants [2–5]. Only the A subunit has been found in algae. The A and B subunits are very similar, except that the B subunit has a highly negatively charged C-terminal extension that contains two additional cysteine residues. This extension is responsible for the tendency of the A_2B_2 tetramer to aggregate into the A_8B_8 form [6,7]. The polymerization state of the enzyme is linked to its regulation by dark–light transitions. The A_8B_8 form of GAPDH is considered to be a regulatory one, whose activity *in vitro* may be regulated by metabolites such as NADP(H) or BPGA in the presence of a reducer [7–9]. This regulation is mediated by the dissociation of the 'heavy' form of GAPDH, leading to the formation of a more active tetramer.

Chloroplast GAPDH has also been isolated from both higher plants and algae as part of a multienzyme complex [10–14]. The composition of the complex varies depending on the species, but often seems to be made up of at least phosphoribulokinase, GAPDH and a recently isolated protein, CP12 [15,16]. The sequence of this small nuclear encoded protein is similar to that of the C-terminal extension of GAPDH subunit B.

This report describes an *Escherichia coli* system for the overproduction of the A_4 GAPDH of the green alga, *Chlamydomonas reinhardtii*. The enzymology of chloroplast GAPDHs has not been studied in detail, in contrast to that of cytosolic GAPDHs (EC1.2.1.12) which are involved in glycolysis. In particular, no A_4 tetramer has ever been characterized. This paper describes the kinetic properties of both the native and recombinant A_4 GAPDHs from *C. reinhardtii*. *In vitro* reconstitution experiments with recombinant GAPDH and CP12 were performed. For the first time, we show that the kinetic properties of GAPDH are modified upon association with the small protein CP12.

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Abbreviations: BPGA, 1,3-bisphosphoglycerate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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Experimental procedures

Expression of *C. reinhardtii* chloroplast GAPDH in *E. coli*

The cDNA coding for the transit peptide and A subunit of *C. reinhardtii* chloroplast GAPDH (1.8 kb) was kindly provided by L. E. Anderson in plasmid Bluescript SK (Stratagene). In order to obtain the mature A subunit, the N-terminus of *C. reinhardtii* chloroplast GAPDH was sequenced (Edman method, Institut Pasteur). The initial amino acid residues were EKKIRVAIN. The *Nde*I restriction site and bases recommended for complete cleavage were added just before the codon for the first amino acid residue by PCR (5′-**GGAATTCCATATG**GAGAAGAAGATCCGC-3′), while the *Bam*HI site and the recommended bases (5′-**CGGGATC**CTTACGCCACCCACTTCTTGG-3′) were added just after the stop codon. The 1.1 kb PCR fragment obtained was cloned into the *Nde*I/*Bam*HI sites of the expression vector pET3a (Novagen).

The *C. reinhardtii* GAPDH was expressed in freshly transformed *E. coli* BL21(DE3)pLysS. Bacteria were grown in LB medium with 100 µg·mL^{-1} ampicillin and 34 µg·mL^{-1} chloramphenicol at 37 °C until the D_{600} reached 0.5–0.6. Cultures were then cooled on ice and induction was performed by adding 1 mM isopropyl thio-β-D-galactoside. Expression was performed at 30 °C overnight.

Preparation of soluble proteins

Bacteria were centrifuged (10 000 ***g***) and the pellet was suspended in Procion buffer (50 mM Tris, 2 mM EDTA, 2 mM dithiothreitol, 0.1 mM NAD, pH 8.0), supplemented with 1 µg·mL^{-1} DNase, 1 µg·mL^{-1} RNase, 10 mM $MgCl_2$, 40 µg·mL^{-1} lysozyme, and protease inhibitors (Sigma). Cells were broken by sonication and centrifuged at 27 000 ***g*** for 20 min. The supernatant contained the recombinant *C. reinhardtii* GAPDH.

Purification of *C. reinhardtii* recombinant GAPDH

The crude extract was applied to an affinity column Procion Red (Amersham-Pharmacia, 1.2 cm × 8 cm) previously equilibrated in Procion buffer. The column was washed with Procion buffer containing 5 mM NAD instead of 0.1 mM and then eluted with a 0–15 mM NADP linear gradient (2 × 30 mL). The fractions containing NADPH- and NADH-dependent GAPDH activities were pooled, concentrated and applied to a PD10 column, equilibrated in 30 mM Hepes KOH pH 8.5, 1 mM dithiothreitol and 0.1 mM NAD (buffer A). The proteins were then applied to a DEAE Trisacryl column (1.2 cm × 8 cm) equilibrated in buffer A. The column was eluted with a 0–0.3 M NaCl linear gradient (2 × 30 mL). A small fraction of pure recombinant GAPDH was also collected in the wash out. The purified recombinant GAPDH was stored at −80 °C in 10% aqueous glycerol.

Purification of GAPDH isolated from *C. reinhardtii*

The GAPDH from *C. reinhardtii* (WM3^{-}) cells grown mixotrophically was purified in the presence of 2 mM dithiothreitol to apparent homogeneity as previously described [13]. The purified enzyme was stored at −80 °C in 10% aqueous glycerol.

Determination of recombinant GAPDH molecular mass by gel filtration

The S300 column (2.6 cm × 95 cm) was calibrated using ferritin (440 kDa), catalase (240 kDa), phosphoglucose isomerase (110 kDa), bovine serum albumin (68 kDa), peroxidase (50 kDa) and cytochrome *c* (12.5 kDa). The void volume of the column, determined with dextran blue, was 228 mL.

Enzyme assays and protein measurements

To determine NADH- or NADPH-dependent activities of GAPDH, 1,3-bisphosphoglycerate (BPGA) was synthesized by incubating 66 mM phosphoglyceric acid, 4.5 units phosphoglycerate kinase and 33 mM ATP in a final volume of 1.5 mL at 30 °C for 20 min. The concentration of BPGA in the presence of 0.25 mM NADH was determined using excess rabbit muscle GAPDH and 10 µL of the previous mixture in a final volume of 1 mL. In most cases, BPGA concentration was found to be 12 mM. Kinetic measurements were performed in 50 mM glycyl-glycin, 50 mM KCl, 10 mM Mg^{2+}, 0.5 mM EDTA at pH 7.7 using the concentrations of substrate and cofactors indicated in the main text. All activities were recorded using a Pye Unicam UV2 spectrophotometer. Experimental data were fitted to theoretical curves using Sigma Plot 5.0. One unit is defined as the quantity of enzyme necessary to convert 1 µmol of substrate per min at 30 °C.

Protein concentrations were determined with the Bio-Rad protein dye reagent, using bovine serum albumin as standard.

pH optimum

Three buffers were used: 50 mM Mes/KOH for pH 6.4–6.8, 50 mM Hepes/KOH for pH 6.8–7.5 and 50 mM glycyl-glycine for pH 7.5–8.9. The remaining components were as in the standard assay.

Electrophoresis

SDS/PAGE (12% acrylamide) was carried out in a Bio-Rad Mini Protean system. Proteins were stained with Coomassie Brilliant Blue R250.

Native PAGE was performed on 4–15% minigels using the Phastsystem apparatus (Pharmacia). Proteins were transferred on nitrocellulose (0.45 µM, Schleicher and Schüll) by passive diffusion. The membranes were immunoblotted against spinach CP12 and *Synecchocystis* GAPDH antibodies. The blots were developed using alkaline phosphatase [17].

Mass spectrometry

MALDI-time of flight (TOF) mass spectra were obtained on a Voyager DE Pro mass spectrometer (Applied Biosystems). Samples were desalted on C_{18} zip tips (Millipore) and eluted in 50% acetonitrile/0.1% trifluoroacetic acid and 50% water/0.1% trifluoroacetic acid. Recombinant and native GAPDHs were analyzed using sinapinic acid (3,5-dimethoxy-4-hydroxycinnaminic acid) as matrix; α-cyano-4-hydroxycinnamic acid was used to analyze CP12.

In vitro recombinant GAPDH/CP12 complex reconstitution

To remove dithiothreitol, recombinant GAPDH was dialyzed in 30 mM Tris, 100 mM NaCl, 2 mM EDTA, 0.1 mM NAD (buffer B) supplemented with 5 mM Cys, pH 7.9. Oxidized CP12 (details of purification to be published elsewhere) was added in different proportions as indicated in the main text. Both proteins were dialyzed in buffer B and concentrated together to a final volume of 50 µL. After concentration, 10% glycerol was added and the proteins were incubated 45 min at 30 °C and then kept at 4 °C overnight or longer. After reconstitution, the samples were submitted to a gel filtration (S300, 44.5 × 1 cm) equilibrated in buffer B supplemented with 1 mM dithiothreitol, pH 7.9. The void volume of the column, determined with dextran blue, was 18 mL.

Results

Purification of recombinant *C. reinhardtii* GAPDH

The *E. coli* soluble protein extract was chromatographed on a Procion Red column. The column was washed with 5 mM NAD in Procion buffer to elute specifically NAD-GAPDH of *E. coli*. The recombinant *C. reinhardtii* GAPDH was eluted at 5 mM NADP. Fractions containing both NADH- and NADPH-dependent activities of GAPDH were pooled, concentrated and desalted on a PD10 column. The resulting solution was fractionated on a DEAE Trisacryl column. Most of the recombinant GAPDH was eluted at 110 mM NaCl. The active fractions were pooled and concentrated. SDS/PAGE showed that they contained only GAPDH (Fig. 1). The molecular mass of the recombinant subunit was estimated at 42.5 ± 2.8 kDa.

A 1-L culture of *E. coli* yielded 1 mg of pure GAPDH with a specific activity of 146 ± 11 U·mg^{-1} when NADPH was used as cofactor and a specific activity of 35 ± 5 U·mg^{-1} when NADH-dependent activity was monitored.

Subunit composition of recombinant GAPDH

According to mass spectrometry studies on MALDI-TOF, the mean molecular mass of recombinant *C. reinhardtii* A subunit expressed in *E. coli* was 37072 ± 65 Da, which corresponded to the mass of the A subunit without cleavage of the initial methionine residue (estimated mass of this form: 37012 Da). The presence of the initial methionine residue was also checked by N-terminal sequencing of recombinant GAPDH.

Gel filtration on a S300 column indicated that recombinant GAPDH had a molecular mass of 155 ± 15 kDa which is close to the molecular mass obtained for native GAPDH (152 ± 15 kDa). Thus, recombinant GAPDH is also an A_4 tetramer.

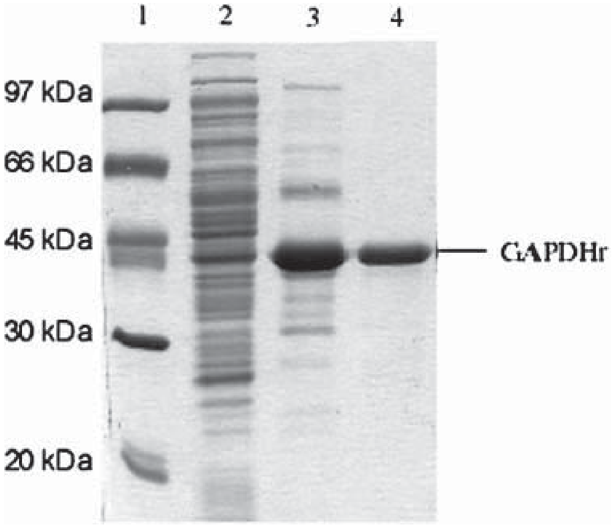

Fig. 1. SDS/PAGE of the purification steps of recombinant *C. reinhardtii* GAPDH. Proteins were separated on 12% polyacrylamide gels under denaturing conditions and stained with Coomassie Brilliant Blue R250. Lane 1, molecular weight markers; lane 2, soluble proteins of the *E. coli* crude extract (15 µg); lane 3, Procion Red pool (10 µg); lane 4, DEAE Trisacryl pool (3.5 µg).

pH optima studies

The NADPH- and NADH-dependent activities of the native and recombinant GAPDHs were tested at pHs from 6.4 to 8.9. The experimental points were fitted to the following equation [18]:

$$k_{\mathrm{obs}} = \frac{k_{\mathrm{cat}}}{1 + \left(\frac{[\mathrm{H}^+]}{K_{\mathrm{a}}}\right) + \left(\frac{K_{\mathrm{b}}}{[\mathrm{H}^+]}\right)} \quad (1)$$

where k_{cat} is the estimated catalytic constant, k_{obs} the experimental catalytic constant, and K_{a} and K_{b} the ionizing side chain constant of the residues involved in the catalytic mechanism.

Both enzymes had a broad pH dependency with bell-shaped curves. The pK_{a} and pK_{b} values were estimated (Table 1).

Whatever activity was considered, pK_{a} values were similar and close to the pK value of histidine. The pK_{b} values were also the same for all activities studied, and corresponded to the pK of cysteine.

The pH optimum $\left(\frac{pK_{\mathrm{a}} + pK_{\mathrm{b}}}{2}\right)$of native GAPDH for NADPH-dependent activity was 7.7 ± 0.1, very close to the optimum pH for the recombinant enzyme (7.9 ± 0.1).

Table 1. pK_{a} and pK_{b} values for recombinant and native GAPDH using NADPH or NADH as cofactor.

	pK_{a}	pK_{b}
Recombinant NADPH–GAPDH	6.15 ± 0.14	9.58 ± 0.02
Native NADPH–GAPDH	6.4 ± 0.17	9.03 ± 0.01
Recombinant NADH–GAPDH	6.25 ± 0.12	9.44 ± 0.02
Native NADH–GAPDH	6.17 ± 0.14	9.34 ± 0.01
Cysteine (ionizing side chain)		9.1 – 9.5
Histidine (ionizing side chain)	6.2	

The pH optimum for native and recombinant GAPDH activities with NADH were also similar (7.8 ± 0.1 and 7.9 ± 0.1).

Determination of kinetic parameters of native and recombinant GAPDH

The enzyme activities measured at constant cofactor (NADPH or NADH) concentration (0.25 mM) and varied BPGA concentrations were fitted to a sigmoid:

$$\frac{\mathrm{v}}{[\mathrm{E}]_0} = k_{\mathrm{cat}} \times \left(\frac{[\mathrm{BPGA}]^{n_{\mathrm{h}}}}{K_{0.5}{}^{n_{\mathrm{h}}} + [\mathrm{BPGA}]^{n_{\mathrm{h}}}} \right) \qquad (2)$$

where k_{cat} is the catalytic constant, n_{h} the Hill coefficient and $K_{0.5}$ the BPGA concentration for which half the maximal velocity is obtained.

Thus, the native and recombinant GAPDHs showed allosteric behavior with respect to BPGA whatever the cofactor used (Fig. 2A,B).

The NADPH-dependent catalytic rate constants for native GAPDH (223 ± 9 s^{-1}) were 50% of those for recombinant GAPDH (419 ± 13 s^{-1}). It was also the case for the NADH-dependent catalytic rate constants of native GAPDH (40 ± 0.9 s^{-1}) and recombinant GAPDH (88 ± 4 s^{-1}). The NADPH-dependent activity was always higher than the NADH-dependent activity for both native and recombinant enzymes. The NADPH-dependent $K_{0.5}$ values for recombinant GAPDH (250 ± 17 μM) were also higher than those for the native enzyme (151 ± 13 μM), as were the NADH-dependent $K_{0.5}$ values (95 ± 10 μM for the recombinant form and 45 ± 2 μM for the native form). The Hill coefficients show that cooperativity for BPGA was positive (value near 1.5 for both enzymes), with both cofactors (specific values are given in Table 2).

The steady-state rates of recombinant or native GAPDH with either NADH or NADPH followed Michaelis–Menten kinetics when the BPGA concentration was kept at 850 μM and the NAD(P)H concentration varied from 0 to 300 μM (Fig. 3A,B). The data were fitted to a hyperbola (Eqn 3) to estimate the catalytic constant (k_{cat}) and K_{m}.

$$\frac{\mathrm{v}}{[\mathrm{E}]_0} = k_{\mathrm{cat}} \times \frac{[\mathrm{NAD(P)H}]}{K_{\mathrm{m}} + [\mathrm{NAD(P)H}]} \qquad (3)$$

The catalytic rate constants for native GAPDH (251 ± 9 s^{-1}) were one-half those for recombinant GAPDH (430 ± 17 s^{-1}) when the NADPH concentration was changed, as were the catalytic rate constants when NADH was the cofactor [41 ± 5 s^{-1} (native enzyme) and 104 ± 3 s^{-1} (recombinant enzyme)]. The K_{m} values for NADPH were slightly higher for recombinant GAPDH (28 ± 3 μM) than for native GAPDH (18 ± 2 μM). In order to check if the K_{m} values were significantly different, we fitted the curves for recombinant and native GAPDH with a multifit using a common value of K_{m} and different values of k_{cat}. The estimated parameters had a value of 25 ± 2 μM for the K_{m}, and the k_{cat} for recombinant and native GAPDH were estimated to 416 ± 13 s^{-1} and to 274 ± 11 s^{-1}, respectively. The K_{m} for NADH were quite similar [136 ± 33 μM (native) and 120 ± 11 μM (recombinant)]. A multifit was also performed. The common value of K_{m} was 143 ± 15 μM and the k_{cat} for recombinant and native GAPDH were equal to 114 ± 6 s^{-1} and 42 ± 3 s^{-1}, respectively. The distribution of the residuals for individual and multifits did not significantly differ (data not shown).

The catalytic efficiencies or specific constants ($k_{\mathrm{cat}}/K_{\mathrm{m}}$) for recombinant ($1.5 \times 10^7$ $\mathrm{M}^{-1}\cdot\mathrm{s}^{-1}$) and native ($1.4 \times 10^7$ $\mathrm{M}^{-1}\cdot\mathrm{s}^{-1}$) GAPDH were similar when NADPH was cofactor. They were slightly higher for recombinant GAPDH (9×10^5 $\mathrm{M}^{-1}\cdot\mathrm{s}^{-1}$) than for the native enzyme (3×10^5 $\mathrm{M}^{-1}\cdot\mathrm{s}^{-1}$) when NADH was used as cofactor.

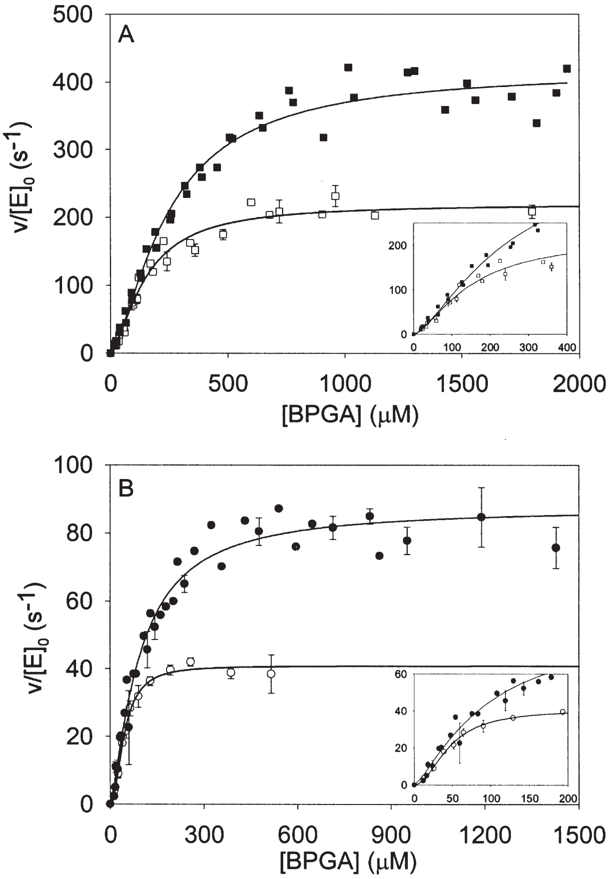

Fig. 2. Steady-state kinetics of recombinant and native GAPDH with varying concentrations of BPGA. (A) Recombinant GAPDH (final concentration of 1.5×10^{-9} M, ■) and native GAPDH (6×10^{-9} M, □) were placed in the reaction mixture containing 0.25 mM NADPH with BPGA concentrations of 0–1.8 mM and the appearance of products was monitored. The initial velocities were determined and the rate constants of three experiments are reported as a function of BPGA concentration. All the experimental points were fitted to a sigmoid (Eqn 2 in the main text). Detailed fitting of the first points is given in the inset. (B) Recombinant (3×10^{-9} M, ●) and native (1.8×10^{-8} M, ○) GAPDH were placed in a NADH-dependent GAPDH assay mixture containing 0.25 mM NADH and BPGA concentrations of 0–1.4 mM. Mean rate constants and their corresponding standard deviations are reported as a function of BPGA concentration. The experimental points were also fitted to a sigmoid (Eqn 2 in the main text). The sigmoid shape of the curve is detailed in the inset.

Table 2. Kinetic parameters of native and recombinant GAPDH.

GAPDH		n_h	BPGA $K_{0.5}$ (μM)	BPGA k_{cat} (s^{-1})	NAD(P)H K_m (μM)	NAD(P)H k_{cat} (s^{-1})
Recombinant	NADPH	1.5 ± 0.1	250 ± 17	419 ± 13	28 ± 3	430 ± 17
	NADH	1.3 ± 0.1	95 ± 10	88 ± 4	120 ± 11	104 ± 3
Native	NADPH	1.5 ± 0.2	151 ± 13	223 ± 9	18 ± 2	251 ± 9
	NADH	2.1 ± 0.2	45 ± 2	40 ± 1	136 ± 33	41 ± 5

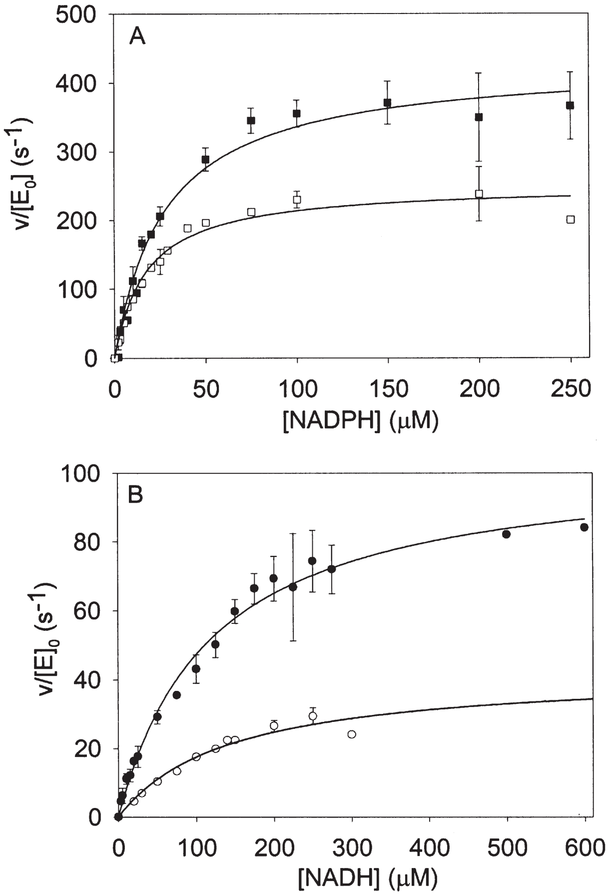

Fig. 3. Steady-state kinetics of recombinant and native GAPDH with varying concentrations of NAD(P)H. (A) NADPH concentration varied from 0 to 250 μM, while BPGA concentration was kept at 0.85 mM. Recombinant (■) and native (□) GAPDH (1.5×10^{-9} M and 6×10^{-9} M, respectively) were placed in the assay cuvette and the appearance of product was monitored. Mean rate constants and standard deviations are reported as a function of NADPH concentration in the assay cuvette. The points were fitted to a hyperbola (Eqn 3 in the main text). (B) The NADH-dependent activity of recombinant (●) and native (○) GAPDH (3×10^{-9} M and 1.8×10^{-8} M, respectively) was monitored with the NADH concentration at 0–600 μM and the BPGA concentration kept at 0.85 mM. The mean rate constants and standard deviations are reported as a function of NADH concentration. The experimental points were fitted to a hyperbola (Eqn 3 in the main text).

The average NADPH- to NADH-linked activity ratios were 4.8 ± 0.8 for the recombinant enzyme and 6.0 ± 0.4 for the native GAPDH.

MALDI-TOF analysis of native GAPDH

Studies of native GAPDH by MALDI-TOF mass spectrometry gave a mass spectral peak at *m/z* 36 854 Da (estimated value 36 881 Da) and at 8509 Da. The first peak corresponded to the estimated mass of the A subunit. Thus, the GAPDH from *C. reinhardtii* copurified with a small protein of 8509 Da. This protein is absent from the recombinant GAPDH sample.

Wedel and Soll [16] showed that *C. reinhardtii* GAPDH could be part of a multienzyme complex composed of phosphoribulokinase, GAPDH and a small 8.5 kDa protein, CP12. A 8.5-kDa protein was also found in the complex described by Avilan *et al.* [13,19–24] by mass spectrometry, showing that this complex also contained CP12. When GAPDH was dissociated from phosphoribulokinase by reduction with 20 mM dithiothreitol for 1 h at 30 °C and then submitted to a gel filtration (S300) in the presence of 5 mM dithiothreitol, GAPDH still copurified with CP12. Thus, the gel filtration and mass spectrometry results indicate that native GAPDH is a complex of GAPDH (152 ± 15 kDa) with CP12. This complex is stable, even in the presence of dithiothreitol, up to 20 mM.

Recombinant GAPDH and CP12 reconstitution experiments

To check whether the different kinetic parameters obtained for native and recombinant GAPDHs were linked to the presence of CP12 with native GAPDH, reconstitution experiments were performed using different molar proportions of GAPDH:CP12 (1 : 1; 1 : 2; 1 : 4).

After incubation during 14 h at 4 °C, a native PAGE was performed and a new band appeared in the presence of CP12 (Fig. 4). This band was recognized by both CP12 and GAPDH antibodies. Samples incubated 45 min at 30 °C or 14 h at 4 °C were submitted to a gel filtration and the fractions containing GAPDH activity were pooled and concentrated. GAPDH eluted at a volume of 26 mL whereas isolated CP12 eluted at 36 mL. SDS/PAGE gels showed that CP12 copurified with GAPDH (data not shown).

$K_{0.5}$ for BPGA, using NADPH as cofactor was first determined after 45 min at 30 °C. The k_{cat} of the reconstituted GAPDH/CP12 complex decreased and was equal to that obtained with native GAPDH, but the $K_{0.5}$ value remained equal to that of recombinant GAPDH (Fig. 5). After 14 h at 4 °C, kinetic experiments showed that the k_{cat} of the reconstituted complex was still equal to the k_{cat} of native GAPDH and the $K_{0.5}$ for BPGA also became equal to that of native GAPDH. Control experiment (GAPDH

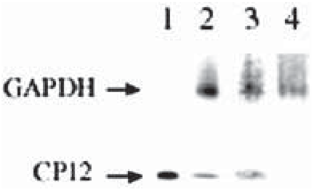

Fig. 4. Western blot analysis of the *in vitro* reconstitution of the recombinant GAPDH/CP12 complex. Aliquots from the reconstitution mixture were separated on a 4–15% gradient native gel. The gel was stained with Blue Coomassie (2). The proteins were also transferred on a nitrocellulose membrane and immunoblotted against antispinach CP12 (given by N. Wedel) (1, CP12 alone; and 3, reconstitution mixture) and anti-*Synecchocystis* GAPDH (given by Valverde) antibodies (4, reconstitution mixture). We checked that CP12 antibodies did not cross-react with recombinant GAPDH.

alone) showed no changes and the kinetic changes were specifically linked to the association of CP12 with recombinant GAPDH.

Discussion

We have developed an overexpression system in *E. coli* that provides large quantities of *C. reinhardtii* GAPDH and allowed us to develop a purification procedure that is simpler than that used for GAPDH extracted from the green alga. Mass spectrometry and N-terminal sequencing of recombinant GAPDH indicate that the initial methionine residue has not been cleaved in *E. coli*. The molecular mass obtained by gel filtration indicates that recombinant GAPDH is a homotetramer of A subunits, as expected.

The pH optima of native and recombinant GAPDH are similar for both NADH- and NADPH-dependent activities. GAPDH has a pH optimum near 7.8. Nevertheless, GAPDH has a broad pH dependency and small changes in pH over the physiological range of 7.0–8.0 have little effect on the activity of the enzyme. Although the pH in the stroma increases from 7.0 to 8.0 upon dark to light transitions [25], this does not seem to play a major role in the regulation of the A_4 tetramer of GAPDH.

Moreover, if the enzyme is considered as a dibasic acid (EH_2), by fitting the experimental points obtained at different pH to Eqn 1, the pK_a and pK_b corresponding to the two nonidentical acidic groups involved in catalysis may be determined. The values obtained (approximately 6.2 and 9.3) are close to the theoretical pK values of histidine (6.2) and cysteine (9.1–9.5) [26]. The Cys149 in glycolytic GAPDH is involved in the formation of the hemithioacetal intermediary during catalysis, while His176 may interact with Cys149 through a hydrogen bond [27]. By extension, the results for chloroplast GAPDH seem to indicate that the equivalent amino acid residues (Cys156 and His183 in *C. reinhardtii* sequence) take part in catalysis.

We have also determined the kinetic parameters of an A_4 tetramer of GAPDH for the first time. Kinetic studies on the A_8B_8 and A_2B_2 forms of spinach, *Synechococcus* PCC 7942 and *Sinapis alba* GAPDH are the only published data on native chloroplast GAPDH [4,28–30]. When the BPGA concentration was held constant, and NAD(P)H concentrations varied, the catalytic activity of native *C. reinhardtii* GAPDH followed Michaelis–Menten kinetics, as do other NADPH–GAPDHs. The values of the K_ms ($K_{mNADPH} = 18 \pm 2$ µM and $K_{mNADH} = 120 \pm 11$ µM) are also similar to those found in the literature (Table 3).

When cofactor concentration was held constant and BPGA concentration changed, the native *C. reinhardtii* GAPDH exhibited a positive cooperativity towards BPGA, with a Hill coefficient of about 1.5. In contrast, other NADPH–GAPDHs follow Michaelis–Menten kinetics towards BPGA. Kinetic studies on a recombinant B_4 tetramer and a B_4 tetramer with a B subunit lacking its C-terminal extension (gapB$^{\Delta C}$), show that these forms also have Michaelis–Menten kinetics [7,31]. The results for the gapB$^{\Delta C}$ are rather surprising, as the truncated B subunit is very similar to the *C. reinhardtii* A subunit, and so, should behave similarly. Thus, the positive cooperativity of *C. reinhardtii* GAPDH is a specific property of this enzyme. This behavior might be physiologically relevant, as BPGA is believed to be the most likely cause of light activation of GAPDH *in vivo* [7]. This cooperativity is all the more important as the regulatory form A_8B_8, which is regulated by BPGA in higher plants, does not exist in the green alga and as the A_4 GAPDH of *C. reinhardtii* is not activated by BPGA [32].

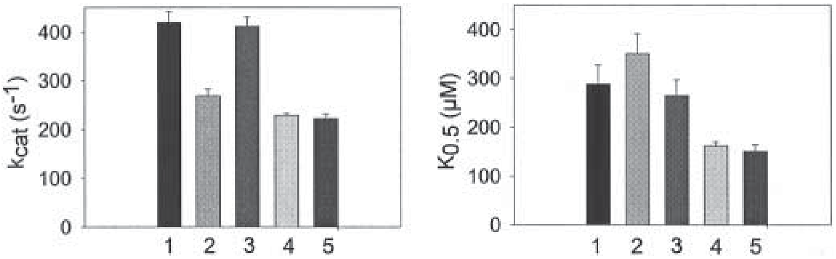

Fig. 5. Kinetic changes of recombinant GAPDH upon association with CP12. GAPDH was incubated with CP12 in a molar ratio of 1 : 2 (0.2 nmol GAPDH and 0.4 nmol CP12). After 45 min at 30 °C or incubation 14 h at 4 °C, the kinetic parameters of GAPDH incubated with CP12 or not (control) were determined using varying concentrations of BPGA, while NADPH concentration was held constant at 0.25 mM. The experimental points were fitted to Eqn (2). The estimated parameters and their standard errors are reported in the histogram. The mean values and the mean standard errors of native GAPDH are also reported. After 45 min at 30 °C: 1, control; 2, incubation with CP12. After 14 h at 4 °C: 3, control; 4, in the presence of CP12; 5, native GAPDH.

Table 3. Kinetic parameters of various NADPH–GAPDHs. Kinetic studies on spinach, *S. alba*, and *Synechococcus* PCC 7942 were from [28–30]. ND, not done.

GAPDH		K_{mBPGA} (μM)	$K_{\mathrm{mNAD(P)H}}$ (μM)	AS ($\mathrm{U \cdot mg^{-1}}$)
Spinach A_8B_8	NADPH	> 100	30–60	ND
	NADH	35 ± 8	120–290	43
Spinach A_2B_2	NADPH	20	30–60	138
	NADH	16 ± 10	120–290	58
S. alba A_2B_2	NADPH	ND	23	120–150
	NADH	ND	300	ND
Synechococcus PCC 7942	NADPH	ND	62 ± 4.5	150
	NADH	ND	420 ± 11	27

Besides the different behaviors towards the substrate, the K_m or $K_{0.5}$ values of *C. reinhardtii* GAPDH and other NADPH–GAPDHs are different (Table 3). The difference between the A_2B_2 form and *C. reinhardtii* A_4 tetramer is probably due to the different methods used to determine BPGA concentration.

Finally, recombinant and native *C. reinhardtii* GAPDHs both show Michaelis–Menten kinetics with their cofactors (NADPH or NADH). Using a multiple function nonlinear regression, we show that the K_m values for recombinant and native GAPDHs do not differ for NADH and also for NADPH.

The catalytic efficiencies, or specific constants for NADPH- and NADH-dependent activities were quite similar for recombinant and native GAPDH. The obtained values show that chloroplast GAPDH is much more specific for NADPH than for NADH (≈ 17-fold).

Native and recombinant enzymes exhibit the same cooperative behavior towards BPGA, but the $K_{0.5}$ for BPGA and the catalytic constants differ. Mass spectrometry studies revealed that native GAPDH is a complex of GAPDH plus the small protein CP12 (8.5 kDa). This major difference with recombinant GAPDH could explain the different kinetic properties obtained. Yet, an effect of the initial methionine residue or folding problem in *E. coli* cannot be ruled out.

To discriminate between these hypotheses, *in vitro* reconstitution assays were performed. They show that upon association of CP12 with GAPDH, the kinetic parameters of the latter change in a two-step process to finally become identical to those of native GAPDH. The decrease of the catalytic constant is a fast process compared to the decrease of the $K_{0.5}$ for BPGA. These changes are most likely linked to conformational changes in the GAPDH/CP12 complex.

These results are a first step towards the understanding of the role of CP12 and this point is currently under investigation.

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