The non-regulatory isoform of NAD(P)-glyceraldehyde-3-phosphate dehydrogenase from spinach chloroplasts

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Abstract

Isoforms of NAD(P)-dependent glyceraldehyde-3-phosphate dehydrogenases (EC 1.2.1.13) have been separated from spinach chloroplast extracts by FPLC-anion exchange chromatography in phosphate buffer and purified to homogeneity. Peak I from Q-Sepharose corresponds to a tetramer of A-subunits of 36 kDa showing a constant ratio of NADPH- to NADH-activity of 2 (insensitive to substrate-modulators), and is defined as A₄ or non-regulatory isoform (GAPDHₙ). GAPDHₙ always amounts to 15–20% of total enzyme regardless of the purification procedure. A small peak II in Q-Sepharose eluates gives rise to 300 kDa and 150 kDa species. Peak III isoform from Q-Sepharose corresponds to the well-known regulatory NAD(P)-glyceraldehyde-3-phosphate dehydrogenase oligomer (GAPDHᵣ) and contains equimolar quantities of 36 kDa (A) and 39 kDa (B) subunits. Following storage of GAPDHᵣ under reducing conditions, partial degradation of B-subunits occurred, affecting the quaternary structure of the active enzyme.

Steady-state kinetics of GAPDHₙ have been studied at pH 7.5. The patterns are consistent with the general reaction mechanism of glyceraldehyde-3-phosphate dehydrogenases and feature high Kᵦₛ, and substrate inhibition responses with increasing glyceraldehyde-3-phosphate or phosphate. The Vₘₐₓ values of reactions with either NADP⁺ or NADPH at saturating concentrations of all substrates are similar, and 2.5-fold higher than for reactions using NAD⁺ or NADH. Haldane relationships result in Kₑقار = 4.6 × 10⁻² M, the experimentally derived value being Kₑ orderby = 16 × 10⁻² M. The kinetic responses of GAPDHᵣ in the aggregated state (600 kDa) were identical to those of GAPDHₙ, except that Vₘₐₓ with NADP(H) was 8-fold lower on a protein basis. The kinetic data are consistent with a GAPDHᵣ model where B-subunits are mostly responsible for regulatory effects and A-subunits for catalysis.

Key words: Glyceraldehyde-3-phosphate dehydrogenase, C₃ cycle, NADPH, isoenzymes, chloroplast.

Introduction

Chloroplast glyceraldehyde-3-phosphate dehydrogenase (NAD(P)-GADPH; EC 1.2.1.13) catalyses with phosphoglycerate kinase the conversion of PGA, the product of photosynthetic carboxylation, to triose phosphate. The coupled reaction consumes most of the initial photosynthetic products NADPH and ATP and is metabolically regulated by light (Cseke and Buchanan, 1986).

The regulatory chloroplast enzyme (GAPDHᵣ) is formed by subunits A (36 kDa) and B (39 kDa) in stoichiometric amounts. The primary structures of the two subunits show considerable homology and the respective nuclear genes (GapA and GapB) have been investigated in algae and higher plants (Cerf, 1995). By contrast, the glycolytic glyceraldehyde-3-phosphate dehydrogenases (NAD-dependendent), GapC gene products, are trimers of 37 kDa subunits. Glycolytic NAD-GAPDH is active in the cytoplasm in all eukaryotic cells, although products of a newly discovered gene subfamily GapCp are targeted to plastids in some plants (Meyer-Gauen et al., 1994).

Chloroplast GAPDHᵣ occurs in vitro as a 600 kDa regulatory form (A₈B₈) which shows high NAD(H)-dependent and low NADP(H)-dependent activities. Several substrates-effectors and hydrophobic or choa-
tropic compounds promote the dissociation of A₈B₈ oligomers to A₂B₂ protomers having high NAD(P)H activity (Pupillo and Faggiani, 1979; Wolosiuk and Stein, 1990; Baalmann et al., 1995). This process underlies the light-induced increase of NADPH-dependent GAPDH activity in planta known since 1965 (Ziegler and Ziegler, 1965).

Leaves, however, may contain other, less abundant NAD(P)-GAPDH species (Wolosiuk and Buchanan, 1976) and most preparations showed an excess of A-subunits (Pupillo and Faggiani, 1979). Evidence was found for an A₄ isoform besides the major A/B species (Cerff and Chambers, 1979; Iadarola et al., 1983). The present study reports on the purification and biochemical characterization of the A₄ isoform from isolated spinach chloroplasts. The native A₄ form is defined as non-regulatory (GAPDH₄⁺) since it fails to aggregate into larger oligomers and is insensitive to modulators of the major A/B-type GAPDH₄⁻ but its kinetic properties are otherwise similar to GAPDH₄⁻. The study of GAPDH₄⁺ may thus help unravel some unexplained features of this important chloroplast enzyme.

Materials and methods

Chloroplasts

Spinach leaves (usually in batches of 500 g) were rinsed, cut to pieces, and homogenized at medium speed (2 × 20 s, 0–2 °C) in the presence of 50 mM K-phosphate pH 7.5, 1 mM PMSF, 2 mM Na₂EDTA, and 10 mM 2-mercaptoethanol (buffer A) plus 300 mM sucrose, at the ratio of 2 ml buffer g⁻¹ leaves. Chloroplasts were sedimented (3000 g, 5 min), gently resuspended with homogenization buffer to 1/10 of the initial volume and precipitated again. The washed chloroplast sediment, containing 15–20% of the original NADPH-dependent activity of the homogenate, was suspended in buffer A and crystalline ammonium sulphate was then dissolved to 40% saturation. The clarified supernatant (20 000 g, 20 min) was used as enzyme source.

Enzyme purification

The enzyme was purified from chloroplast supernatant, containing 40% saturated ammonium sulphate. Further ammonium sulphate was added to 80% saturation and the precipitate (20 000 g, 20 min) was resuspended in buffer A to 25 ml. An equal volume of acetone at −20 °C was poured with mixing and the precipitated protein (20 000 g, 30 min, −20 °C) was dissolved in 10 ml of buffer A, then stored overnight at −20 °C.

The preparation was now cleared by centrifugation and desalted through a Sephadex G-25 column (HiTrap, Pharmacia) equilibrated with buffer B (25 mM K-phosphate, 1 mM Na₂EDTA, 10 mM 2-mercaptoethanol, pH 7.5). Proteins were loaded on a 1.6 × 10 cm Q-Sepharose High Performance column in the FPLC system (Pharmacia) equilibrated with this buffer. After 20 ml washing a 100 ml linear K-phosphate gradient (25–500 mM, pH 7.5, containing 1 mM Na₂EDTA and 10 mM 2-mercaptoethanol) was developed at 1 ml min⁻¹. Two main peaks of NAD(P)H-dependent activity were found at 150 and 330 mM K-phosphate. These proteins, as shown in the Results, correspond to GAPDH₄⁺ and GAPDH₄⁻, respectively.

The two NAD(P)-GAPDH peaks were separately concentrated by ultrafiltration (Amicon PM10 membrane), equilibrated by gel filtration (HiTrap, Pharmacia) with buffer C (50 mM Tris-HCl, pH 7.5, 1 mM Na₂EDTA, 10 mM 2-mercaptoethanol) and incubated in the presence of 1 mM NAD⁺ for 1 h at 4 °C. The two forms are then loaded on a Superdex–200 column (HiLoad 1.6 × 60 cm, FPLC) equilibrated with buffer C plus 150 mM KCl and 0.1 mM NAD⁻. GAPDH⁺ was further purified by adsorption to a 2'-5'-ADP Sepharose 4B column (1 × 10 cm) equilibrated in buffer A without PMSF. After 25 ml washing the GAPDH⁺ was eluted by adding 5 ml of 0.25 mM NAD⁺ in buffer A. On the other hand, final purification of GAPDH⁻ was achieved following buffer exchange to buffer C plus 1 mM NAD⁺ instead of NAD⁻, incubation for 1 h at 4 °C and passage through Superdex 200 equilibrated with buffer C plus 150 mM KCl and 0.1 mM NAD⁺. Recoveries were usually close to 100% in each chromatographic step. Similar purification protocols have also been applied to total leaf homogenates, with the drawback that final GAPDH⁺ preparations were often contaminated by another polypeptide.

Purified GAPDH⁺ was stored under nitrogen in buffer C plus 0.05 mM NAD⁺ and 2 mM DTT at 4 °C. Purified GAPDH⁻ was stored in vials at 4 °C in the presence of buffer C plus 1.0 mM NAD⁻ (A₄B₂ form); when needed the A₂B₂ form was then obtained by exchanging NAD⁻ to 1 mM NAD⁺ or 50 µM BPGA by means of Sephades G-25 column desalting.

Other methods

Reverse-phase chromatography of purified GAPDH⁺ was performed under denaturing conditions on a Sephasil Peptide C18 column (ST 4.6/250, Pharmacia) connected to a Smart System (Pharmacia). Pure GAPDH⁺ (20 µg) was treated with 1% (w/v) SDS, 1% (v/v) β-mercaptoethanol, and 1% (v/v) glycerol at 50 °C for 5 min. The column was equilibrated with solvent A (0.065% (v/v) trifluoroacetic acid, 2% (v/v) acetonitrile, in water). Elution was performed with a discontinuous gradient (50–70% solvent B: 0.05% (v/v) trifluoroacetic acid in acetonitrile). Protein was optically detected at 220 and 280 nm.

SDS-PAGE was performed using 12.5% Excelgel SDS containing 15–20% of the original NADPH-dependent activity of the homogenate. The study of GAPDH₄⁺ may thus help unravel some unexplained features of this important chloroplast enzyme.

Enzyme activity measurements

Standard NAD(P)-GAPDH activity assay was performed at 25 °C in 50 mM TRIS-HCl, 1 mM Na₂EDTA, 3 mM PGA, 5 mM MgCl₂, 2 mM ATP, 5 µM iPGK, 0.5 mM KCN (pH 7.5), with 0.2 mM NADPH or NADH as specified. Oxidation of NAD(P)H was followed at 340 nm in an Uvikon 941 plus spectrophotometer (Kontron, Italy).

For steady-state kinetic analysis, initial velocities (0–30 s) were measured in 50 mM TRIS-HCl, 1 mM Na₂EDTA (pH 7.5) at 25 °C. Reductive reaction (NAD(P)H oxidation) was measured with variable concentrations of NAD(P)H and BPGA. Oxidative reaction (NAD(P)⁺ reduction) was assayed by varying NAD(P)⁺, G3P and K-phosphate, but 0.05 mM ADP, 2 mM MgCl₂ and 5 U ml⁻¹ PGK were also added to force GAPDH equilibrium towards BPGA formation (Duggleby and Dennis, 1974). Data points were processed by non-linear regression analysis as in Spanel et al. (1996).

Equilibrium constant (Kₑ) was determined in the medium of kinetic experiments (50 mM TRIS-HCl, 1 mM Na₂EDTA, pH 7.5) at 25 °C, by measuring the NADPH formed at 340 nm in a Uvikon 941 plus spectrophotometer.
equilibrium by GAPDH\textsubscript{N} and 0.3 mM G3P, 2.3 mM K-phosphate, 0.7 mM NADP\textsuperscript{+} (initial concentrations).

**Results**

**Purification of two NAD(P)-GAPDH isoforms**

Protein preparations obtained from washed spinach chloroplasts were enriched by ammonium sulphate and acetone fractionation, desalted and subjected to FPLC anion-exchange chromatography in a Q-Sepharose column using a K-phosphate gradient (Fig. 1). Two main NAD(P)-GAPDH peaks were seen in eluates, respectively at 150 mM (about 20% of total activity, peak I) and 330 mM K-phosphate (about 70% of the activity, peak III), with an intervening smaller peak II. Under these conditions the NADPH-dependent activity associated with each peak was maximal and exceeded the NADH-activity by 2-3 times (not shown), since phosphate acts as an activating substrate for GAPDH\textsubscript{R} (Pupillo and Giuliani Piccari, 1975). The K-phosphate buffer was essential for full preservation of both NADPH- and NADH-dependent enzyme activities at this step. Yields with other buffers (TRIS, Tricine, Tea, pyrophosphate) were consistently lower. In these experiments, the NAD(P)-GAPDH isoforms resolved in Fig. 1 were not separated by ammonium sulphate fractionation as reported (Cerff and Chambers, 1979).

Enzyme peaks I, II, and III were further investigated by gel permeation chromatography in Superdex 200 column (FPLC) in the presence 0.1 mM NAD\textsuperscript{+}, since NAD\textsuperscript{+} specifically promotes the association of most of the enzyme into a 600 kDa ‘NADP-suppressed’ conformation (Pupillo and Giuliani Piccari, 1973). However, the peak I isoform under these conditions exhibited a molecular mass of 150 kDa and high NADPH-dependent activity relative to the NADH-dependent activity (Fig. 2A). Peak I isoform, therefore, was defined non-regulatory or GAPDH\textsubscript{N}. The intermediate peak II was resolved into distinct 300 kDa and 150 kDa species under analogous conditions (Fig. 2B). Peak III, the major NAD(P)-GAPDH isoform (Fig. 2C), had a mass of 600 kDa and low NADPH-activity as expected, corresponding to the regulatory oligomer of Wolosiuk and Buchanan (1976) namely GAPDH\textsubscript{R}.

GAPDH\textsubscript{R} was finally purified by ADP-Sepharose chromatography using NADP\textsuperscript{+} as eluant (Table 1). GAPDH\textsubscript{R} was incubated, instead, with 1 mM NADP\textsuperscript{+} to promote dissociation of A\textsubscript{2}B\textsubscript{2} GAPDH\textsubscript{R} into A\textsubscript{2}B\textsubscript{2} protomers (Pupillo and Giuliani Piccari, 1975) and chromatographed free of contaminants by a second Superdex 200 gel filtration in the presence of K-phosphate and 0.1 mM NAD\textsuperscript{+} (not shown). The purity of the preparations was checked by SDS-PAGE (Fig. 3, lanes B, C). Based on maximum NADPH-dependent activity following GAPDH\textsubscript{R} activation with BPGA (Table 2), the specific activities of both isoforms were similar (about 120 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) protein).

**Properties of GAPDH\textsubscript{N}**

Purified GAPDH\textsubscript{N} migrated as single band of approximately 41 kDa on SDS-gel electrophoresis, corresponding to the

![Fig. 1. Anion-exchange chromatography of NAD(P)-GAPDH isoforms. Desalted enzyme from chloroplast supernatant, enriched by ammonium sulphate and acetone fractionation (30 mg protein), was chromatographed in a Q-Sepharose column (FPLC, 1.6 x 10 cm) equilibrated with 25 mM K-phosphate, 1 mM Na\textsubscript{2}EDTA, 10 mM 2-mercaptoethanol (pH 7.5). Linear K-phosphate gradient in buffer (25 to 500 mM, 100 ml), fractions of 0.25 ml. Three peaks of NAD(P)-dependent GAPDH activity were detected: peak I (fractions 21–23), peak II (fractions 30–33), and peak III (fractions 37–41).](https://academic.oup.com/jxb/article-abstract/49/325/1307/506269/1309)

![Fig. 2. Gel filtration of NAD(P)-GAPDH isoforms. Elution profiles of NADPH- (○) and NADH-GAPDH activity (●) from a Superdex 200 column (FPLC, 1.6 x 60 cm) equilibrated with 50 mM Tea-HCl, 1 mM Na\textsubscript{2}EDTA, 10 mM 2-mercaptoethanol, 150 mM KCl, and 0.1 mM NAD\textsuperscript{+} (pH 7.5). (A) Peak I of Fig. 1 (GAPDH\textsubscript{N}) was desalted and incubated with 1 mM NAD\textsuperscript{+} for 1 h before loading on the column. Molecular mass of NAD(P)-GAPDH forms are indicated on top. (B) Peak II, otherwise as in (A). (C) As in (A), peak III (GAPDH\textsubscript{R}).](https://academic.oup.com/jxb/article-abstract/49/325/1307/506269/1309)
Table 1. Purification scheme of GAPDH<sub>N</sub>

Data from a typical preparation from 0.5 kg of spinach leaves.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Activity (µmol min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Protein (mg)</th>
<th>Specific Activity (µmol min&lt;sup&gt;-1&lt;/sup&gt; mg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Purification (±fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>2365</td>
<td>5760</td>
<td>0.41</td>
<td>0.44</td>
<td>100</td>
</tr>
<tr>
<td>Chloroplasts</td>
<td>425</td>
<td>386</td>
<td>1.1</td>
<td>0.57</td>
<td>18</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>375</td>
<td>155</td>
<td>2.4</td>
<td>0.87</td>
<td>16</td>
</tr>
<tr>
<td>Acetone</td>
<td>320</td>
<td>30</td>
<td>11</td>
<td>3.7</td>
<td>13</td>
</tr>
<tr>
<td>Q-Sepharose (peak I)</td>
<td>56.7</td>
<td>4.8</td>
<td>12</td>
<td>4.4</td>
<td>24</td>
</tr>
<tr>
<td>Superdex 200</td>
<td>48.2</td>
<td>1.5</td>
<td>32</td>
<td>11</td>
<td>78</td>
</tr>
<tr>
<td>ADP-Sepharose</td>
<td>43.7</td>
<td>0.36</td>
<td>121</td>
<td>41</td>
<td>295</td>
</tr>
</tbody>
</table>

*Referred to the NADPH-dependent activity.

Table 2. Effects of substrate-modulators on NADPH-dependent activity of GAPDH<sub>N</sub> and GAPDH<sub>R</sub>

NAD(P)-GAPDH forms were incubated with substrate-modulators at the concentration reported for 10 min at 25 °C. Activity was measured in 50 mM TRIS–HCl, 1 mM EDTA, pH 7.5, 0.2 mM NADPH, 0.05 mM BPGA with aliquots of preincubated enzyme (50-fold diluted in the assay mixture).

<table>
<thead>
<tr>
<th>Substrate-modulator</th>
<th>Concentration (mM)</th>
<th>GAPDH&lt;sub&gt;N&lt;/sub&gt; (µmol min&lt;sup&gt;-1&lt;/sup&gt; mg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>GAPDH&lt;sub&gt;R&lt;/sub&gt; (µmol min&lt;sup&gt;-1&lt;/sup&gt; mg&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>120</td>
<td>45</td>
</tr>
<tr>
<td>BPGA</td>
<td>0.05</td>
<td>124</td>
<td>113</td>
</tr>
<tr>
<td>NADP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.5</td>
<td>117</td>
<td>103</td>
</tr>
<tr>
<td>NAD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.5</td>
<td>122</td>
<td>21</td>
</tr>
</tbody>
</table>

BPGA and NAD<sup>+</sup> (Table 2). This behaviour sharply contrasts with GAPDH<sub>R</sub>, whose NADPH-dependent activity is increased by NADP<sup>+</sup> or BPGA and suppressed by NAD<sup>+</sup> (Table 2; Trost et al., 1993).

Conversion of GAPDH<sub>R</sub> into a GAPDH<sub>N</sub>-like form

Purified GAPDH<sub>R</sub> treated with DTT and NADP<sup>+</sup> under nitrogen occurs as a permanently activated form with high NADPH-activity (Trost et al., 1993). As an odd feature of this preparation, B-subunits were found to be selectively degraded during storage (Fig. 3, lane D), giving rise to a faster migrating band on SDS-PAGE. Enzyme reassociation into a regular A<sub>8</sub>B<sub>8</sub> conformer upon addition of NAD<sup>+</sup> (and removal of DTT and NADP<sup>+</sup>) was also impaired; broad peaks of about 150 and 300 kDa were produced instead, as illustrated in Fig. 4 (compare Fig. 2B).

Do spinach chloroplasts contain native GAPDH<sub>N</sub>?

Since minor NAD(P)-GAPDH species seem to arise from reassembly of GAPDH<sub>R</sub> subunits (Figs 2A, B; 4), the question of the existence of GAPDH<sub>N</sub> <em>in situ</em> was addressed by a shortened fractionation procedure. Chloroplasts were ruptured in hypotonic K-phosphate medium containing 0.2 mM NAD<sup>+</sup>, i.e., compatible with a stable tetramer of A subunits (A<sub>4</sub>), with ammonium sulphate was dissolved to 40% saturation. Following centrifugation, aliquots of supernatant were directly

Fig. 3. SDS-PAGE of purified GAPDH<sub>N</sub> and GAPDH<sub>R</sub>. Two separate gels are shown (lanes A–C, and lanes D, E). Lane A: molecular mass markers (kDa), about 1 µg each. Lane B: Purified GAPDH<sub>N</sub> (0.7 µg). Lane C: GAPDH<sub>R</sub> (0.7 µg). Lane D: GAPDH<sub>R</sub> after 2 weeks storage at 4 °C in the presence of 50 µM NADP<sup>+</sup> and 2 mM DTT under nitrogen. Lane E: molecular mass markers (kDa), about 1 µg each.

The apparent molecular mass of A subunits of GAPDH<sub>R</sub> (Fig. 3), whose true molecular mass is 36 kDa (Ferri et al., 1990). With the aim of checking the homogeneity of the preparation, denatured GAPDH<sub>N</sub> was chromatographed on a HPLC-reverse phase column under conditions optimized for the separation of A and B subunits. A single symmetrical peak was observed, yielding a 41 kDa band in SDS-PAGE (not shown). The native molecular mass of 150 kDa, unchanged by NAD<sup>+</sup>, is thus compatible with a stable tetramer of A subunits (A<sub>4</sub>). BPGA and NAD<sup>+</sup> (Table 2). This behaviour sharply contrasts with GAPDH<sub>R</sub>, whose NADPH-dependent activity is increased by NADP<sup>+</sup> or BPGA and suppressed by NAD<sup>+</sup> (Table 2; Trost et al., 1993).

Fig. 4. SDS-PAGE of GAPDH<sub>R</sub> and GAPDH<sub>N</sub>. Two separate gels are shown (lanes A–C, and lanes D, E).
applied to a Superdex 200 column (Fig. 5). In addition to the major GAPDH$_N$ of 600 kDa, a secondary 150 kDa peak was also present (GAPDH$_N$, 15% of total NADH activity). The experiment was completed within 2 h. By similar experiments under different conditions we found that spinach chloroplast GAPDH$_N$ invariably amounts to 15–20% of total enzyme activity.

**Kinetics of GAPDH$_N$ and GAPDH$_r$**

The kinetics of the purified enzymes were investigated at pH 7.5 by initial velocity analysis. Since some substrates, BPGA in particular, act as hysteretic activators of GAPDH$_r$, assays were adjusted to prevent the activation, as explained in Materials and methods. Patterns and kinetic parameters for both isoforms (GAPDH$_N$ and GAPDH$_r$) were strikingly similar suggesting that the catalytic mechanisms are identical.

Enzyme responses were linear and parallel in double reciprocal plots when NADPH, NADH or BPGA were the varied substrates for the reductive reaction, consistent with a ping pong mechanism (Segel, 1975). $K_m$ values for corresponding substrates turned out to be similar for each isoform (Table 3). The limiting $K_m$ for GAPDH$_N$ was 10-fold lower with respect to $K_m$ for GAPDH$_r$. Experimental $V_{max}$ for A$_r$-GAPDH$_N$ and A$_r$B$_r$-GAPDH$_r$ forms with NADH as coenzyme was 2.5-fold higher than $V_{max}$ with NADH. In contrast, the $V_{max}$ of the NADPH-dependent reaction of A$_r$B$_r$-GAPDH$_N$ was about 3-fold lower than the NADH-dependent $V_{max}$ (NADP$^+$-suppressed state of GAPDH$_N$).

In the reverse or oxidative reaction using G3P, NADP$^+$ and phosphate as substrates, non-linear double reciprocal plots were often observed as both G3P and phosphate gave apparent substrate inhibition at nonsaturating concentrations of the non-varying substrates (Fig. 6). Appropriate substrate concentration ranges had to be chosen, therefore, in order to calculate the kinetic parameters. A complete kinetic analysis was performed for the A$_r$ isoform (Table 4). The absolute maximum velocity ($V_o$) for the oxidative reaction at substrate saturation (using either NADP$^+$ or NAD$^+$ in the presence of Pi and G3P) was not significantly different from $V_{max}$ of the reductive reaction ($V_r$) measured with the corresponding pyridine nucleotide and BPGA, i.e. $V_o \approx V_r$. The reductive reaction driven by NADPH was found to be competitively inhibited by NADP$^+$ (Table 4). The forms of kinetic responses were all consistent with the general reaction mechanism of glyceraldehyde-3-phosphate dehydrogenases (Fersht, 1984) as shown below:

\[
\begin{align*}
\text{NAD(P)H} & \quad \text{G3P} & \quad \text{BPGA} & \quad \text{Pi} & \quad \text{NAD(P)$^+$} \\
\downarrow & \quad \uparrow & \quad \downarrow & \quad \uparrow & \quad \uparrow
\end{align*}
\]

This interpretation is confirmed by the consistency of Haldane's relationships (Segel, 1975) on the basis of seven independently determined kinetic parameters.

### Table 3. Comparison between kinetic parameters of purified NAD(P)-GAPDH forms in the reductive reaction

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Unit</th>
<th>GAPDH$_N$ (A$_r$)</th>
<th>GAPDH$_r$ (A$_r$B$_r$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (NADPH)</td>
<td>$\mu$mol min$^{-1}$</td>
<td>130</td>
<td>114</td>
</tr>
<tr>
<td>$K_{m}$ (NADPH)</td>
<td>$\mu$M</td>
<td>5.4</td>
<td>50</td>
</tr>
<tr>
<td>$K_{m}$ (BPGA)</td>
<td>$\mu$M</td>
<td>2.0</td>
<td>2.3</td>
</tr>
<tr>
<td>$K_{m}$ (G3P)</td>
<td>$\mu$M</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>$K_{m}$ (Pi)</td>
<td>$\mu$M</td>
<td>112</td>
<td>160</td>
</tr>
</tbody>
</table>
The experimentally determined $K_{eq}$ value of the NAD(P)-GAPDH reaction under identical conditions of temperature, pH and ionic strength was $16 \times 10^{-2} \text{ M}^{-1}$, in good agreement with the calculated $K_{eq}$.

**Discussion**

The light-dark modulation of chloroplast NAD(P)-GAPDH is currently envisioned (Scagliarini et al., 1993; Baalmann et al., 1995) in terms of a photosynthetically competent species of 150 kDa ($A_B^2$) reversibly associating into an $A_B^8$ regulatory oligomer with reduced NADPH-dependent activity. The NADPH-activity can be fully recovered *in vitro* in consequence of dissociation of GAPDH into $A_B^2$ protomers, evoked by thioredoxin and/or several substrates-effectors including NADP(H), ATP and with highest efficiency BPGA (Trost et al., 1993). The possible regulatory effects of multienzyme assembly interactions (Wara-Aswapati et al., 1980; Süß et al., 1993; Anderson et al., 1995) remain to be substantiated.

Early reports about isoforms only constituted by A-subunits (Cerff and Chambers, 1979) assumed that such forms were regulatory oligomers by analogy with GAPDH$_N$, and the peculiar properties of GAPDH$_N$ thus escaped attention. The suspicion that the $A_A^4$ isoform could originate from $A_B$-monomers of the $A_B$ enzyme (Iadarola et al., 1983) was supported by the finding that the isolated $A_A$-polypeptide was one and the same (Ferri et al., 1990).

**Properties of GAPDH$_N$ and relationships to GAPDH$_R$**

This paper describes the isolation and purification of NAD(P)-GAPDH forms from spinach chloroplasts. Anion exchange chromatography (Fig. 1) separates three enzyme peaks. Peak I is the $A_A^4$ isoform, here termed GAPDH$_N$ or non-regulatory since it shows a high ratio of NADPH-dependent to NADH-dependent activity insensitive to modifiers (Table 2), and it retains a 150 kDa mass under conditions (Fig. 2) promoting GAPDH$_R$ association into the $A_B^8$ regulatory form (e.g. addition of NADP$^+$). Peak II contains a mixture of unstable oligomers of 150 and 300 kDa, peak III is the principal isoform GAPDH$_N$ constituted of equimolar A and B subunits.

The possibility that GAPDH$_N$ also contains partially degraded B-subunits is unlikely since its A-subunits appear homogeneous in SDS-PAGE and reverse phase chromatography. Moreover, B-subunits are especially prone to proteolysis (Figs 3, 4; Zapponi et al., 1993)
whereas our preparations are insensitive to a variety of protease inhibitors.

GAPDH, resembles GAPDH down to details of the kinetic mechanism. The maximum velocities of the reductive and the oxidative reaction are similar or identical for all enzyme forms, with the provision that \( V_{\text{max}} \) with NADPH (or NADP\(^+\)) exceeds \( V_{\text{max}} \) with NADH (or NAD\(^-\)) by 2.5-fold. The regulatory A\(_B\)\(_B\) oligomer of GAPDH is an exception as it shows sluggish NADP(H)-activity, i.e. \( V_{\text{max}} \) with NADP(H) of this form amounts to 1/3 or less of the unchanged \( V_{\text{max}} \) with NAD(H). Consequently, the access of NADPH to binding sites of the A\(_B\)\(_B\) conformer seems to be restricted, whereas these sites remain fully pervious to NADH. \( K_{m(\text{NADPH})} \) and \( K_{m(\text{NADP}^+)} \) are not affected and early erroneous reports to the contrary (Pupillo and Giuliani-Piccarri, 1973; Wolosiuk and Buchanan, 1976) were due to activating \( (V_{\text{max}}) \) effects by high concentrations of these coenzymes during assays.

Also, all NAD(P)-GAPDH isoforms exhibit similar patterns of substrate inhibition when either G3P or phosphate is varied. The whole reaction mechanism, thoroughly investigated for the first time in the A\(_A\) form, is basically ping-pong as for cytosolic NAD-GAPDH (Fersht, 1984) and Sinapis NAD(P)-GAPDH (Cerf, 1978). A notable feature of the NAD(P)-GAPDH (Table 3) is the 20-fold higher value of \( K_{m(\text{G3P})} \) compared to glycolytic GAPDHs (Duggleby and Dennis, 1973; Copeland and Zammit, 1994), which sounds appropriate for a photosynthetic enzyme reducing BPGA to triose phosphate.

The kinetic data, therefore, are consistent with the contention that the A-subunits are primarily responsible for catalysis both in GAPDH and GAPDH. The native GAPDH\(_A\)\(_A\)\(_B\)\(_B\) protomer contains only two reactive cysteines and as many exchange sites for pyridine nucleotides and acylphosphate localized in A-subunits.

B-subunits represent a less efficient moiety in catalysis (Zapponi et al., 1983; Baalman et al., 1996) with regulatory functions (Li and Anderson, 1997).

On the other hand, enzyme association into large oligomers clearly demands an interaction between both types of GAPDH subunits, involving a role of the non-catalytic C-terminus domain of B-subunits (Brinkmann et al., 1989; Baalman et al., 1996; Li and Anderson, 1997). When 39 kDa B-subunits of spinach GAPDH were truncated in the C-terminus region by Staphylococcus protease, the modified enzyme exhibited a molecular mass near 140 kDa with retention of some activity but complete loss of regulation (Zapponi et al., 1993; Scheibe et al., 1996). The capability of GAPDH\(_A\)\(_B\) subunits to self-assemble into active homotetramers (A\(_A\) or B\(_B\)) was recently shown in heterologous expression systems (Baalman et al., 1996; Li and Anderson, 1997). Recombinant proteins constituted by B-subunits were able to associate into larger oligomers, whereas A-subunits could only form A\(_A\) species. Tetramers of B-subunits with a deletion of 30 amino acids at the C-terminus were also active, but failed to aggregate.

The selective loss of B-subunits during storage of GAPDH\(_A\)\(_B\)\(_B\) protomers, which might be explained by reductant-dependent chemical cleavage (Wu et al., 1995), has been observed. Minor and unstable enzyme forms of 300 kDa found in preparations from leaves and chloroplasts (Figs 1, 2B, 4; Baalman et al., 1995; Scaglìarini et al., 1993) could arise from asymmetric assemblage of A- and B-subunits (Iadarola et al., 1983).

**GAPDH**\(_N\) and plastid metabolism

Speedy fractionation experiments (Fig. 5) suggest, however, that spinach chloroplasts do contain appreciable amounts of native GAPDH. The occurrence of GAPDH\(_N\) is reported for other photosynthetic and non-photosynthetic tissues and GAPDH-like forms or their precursors are often predominant in seedlings (Cerf and Kloppestech, 1982). According to Dewdney et al. (1993), the NAD(P)-GAPDH gene transcript in dark-grown *Arabidopsis* was exclusively A-type, although transcription of GapA was strongly stimulated by light in this and other species (Park et al., 1996). Expression of GapB genes on the contrary shows an absolute light requirement. The GAPDH\(_N\) of non-photosynthetic plastids might function as a glycolytic source of NADPH.

In agreement with Iadarola et al. (1983) it was estimated that 15–20% of total NAD(P)-GAPDH of spinach leaves is GAPDH\(_N\). This isoform and the residual NADPH-linked activity of GAPDH\(_R\) could account for potential NADPH-dependent rates up to \( > 100 \mu \text{mol h}^{-1} \text{mg}^{-1} \text{chl} \) in darkened leaves (Scaglìarini et al., 1993) although the low chloroplast concentration of NADPH \+ NADH (Heineke et al., 1991) would act as a limiting factor. In illuminated chloroplasts any GAPDH\(_N\) contribution to the photosynthetic BPGA reduction would be blurred within a plentiful NADPH-dependent activity of GAPDH, in excess of full photosynthesis rates (Price et al., 1995). In fact, the NAD(P)-GAPDH reaction is usually not rate-limiting since the enzyme is completely activated in daylight (Scaglìarini et al., 1993), but ribulose 1,5-bisphosphate levels are strictly dependent on actual NAD(P)-GAPDH activity (Fridlyand, 1992; Price et al., 1995).

As previously suggested (Trost et al., 1993), the sophisticated control of carbon fluxes through GAPDH in photosynthetic organs may be aimed at regulating chloroplast reactions during dark/light transitions or under limiting conditions, since the light activation process is antagonized by NAD\(^+\) and glyceraldehyde 3-phosphate (Pupillo and Giuliani Piccarri, 1975). The system of NAD(P)-GAPDH isoforms, GAPDH\(_N\) in particular,
ensures that a basal NADPH-dependent activity persists in darkness as in the case of other light-regulated enzymes (Cseke and Buchanan, 1986), thus affording elevated flexibility of metabolism.

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References


