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Structural and kinetic characterization of NADP-dependent, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase from celery leaves

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Abstract

NADP-dependent, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9) from celery leaves was purified over 1200-fold to a specific activity of 35 units/mg protein, and its kinetic, regulatory and structural properties were characterized. The purified enzyme exhibited a homotetrameric structure with a subunit molecular mass of 54 kDa. A high specificity of the enzyme for the substrates $NADP^+$ ($K_m = 7$ μM) and D-glyceraldehyde-3-phosphate ($K_m = 127$ μM) was observed. Maximal activity was determined at pH 8.5. The purified enzyme was highly unstable, requiring the addition of $NADP^+$ or conditions of high ionic strength in the medium. A hysteretic behavior, with a lag phase of minutes, was observed during activity measurement of the enzyme preincubated in the absence of substrates. The lag was inversely proportional to the protein concentration during preincubation. The hysteretic parameters were affected by the substrates, KCl and mannitol among other compounds. Distinctively, incubation with $NADP^+$ produced a near twofold activation of the enzyme. Results suggest that in alditol producing plants the enzyme plays a key role in the synthesis and partitioning of photoassimilates. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Alditols biosynthesis; Carbon partitioning; Celery leaves; Glyceraldehyde-3-phosphate dehydrogenase; Non-phosphorylating

1. Introduction

Three glyceraldehyde-3-phosphate (Ga3P) dehydrogenases with distinctive properties have been isolated from higher plants. Two of them are phosphorylating enzymes that catalyze the reversible oxidation of Ga3P to 1,3-bisphosphoglycerate by either, utilizing NAD^+ and being localized in the cytosol (EC 1.2.1.12) [1–3]; or being NADP-dependent and localized in the chloroplast [2,4–6]. The third enzyme is the non-phosphorylating Ga3P dehydrogenase (EC 1.2.1.9, named GAPN), which catalyzes the irreversible reaction: $Ga3P + NADP^+ + H_2O \rightarrow 3PGA + NADPH + 2H^+$.

GAPN was first described in photosynthetic cells by Arnon's group [7,8] and then purified and characterized from a number of plants [5,9–11] and green algae [12]. The presence of the enzyme was also described in eubacteria [13] and archaebacteria [14]. After studies on the stereospecificity of the cofactor reduction [15] as well as molecular cloning of the gene coding for the enzyme [16], it becomes clear that GAPN is related not to phosphorylating Ga3P dehydrogenases but to the aldehyde dehydrogenase superfamily [6].

Gibbs and coworkers [17,18] proposed a central role for GAPN as participating in a shuttle system for the export of NADPH (photogenerated) from chloroplasts to cytosol. In this system, reducing

Abbreviations: BSA, bovine serum albumin; GAPN, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; Ga3P, glyceraldehyde-3-phosphate; 3PGA, 3-phosphoglycerate.

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power is indirectly transferred via the interchange of reduced triose-phosphates from the plastid to the cytosol, with the oxidation of Ga3P (mediated by GAPN) resulting in the generation of NADPH in the latter compartment [17,18]. Despite this key function played by the enzyme in photosynthetic organisms, its complete characterization is far from complete. The enzyme has been purified from the leaves of *Beta vulgaris* [5], *Hevea brasiliensis* [9] *Spinacia oleracea*, [11,19] and from the green algae *Chlamydomonas reinhardtii* [12]. However, no information is available on the characterics of the enzyme in plants performing major metabolic processes that require high levels of reducing power in the cytosolic compartment.

Celery is a plant that synthesizes mannitol as a major photosynthetic product and translocated carbohydrate [20,21]. Sugar alcohol synthesis occurs in the cytosol of photosynthetic tissues, thus making the source of reducing power in this compartment a particularly relevant issue in this plant species [20,21]. It has been reported that, in celery, the requirement of NADPH for mannitol synthesis, from mannose-6-P, is supplied by GAPN [20]; thus pointing out the particular importance of this enzyme in the metabolism of polyol producing plants. In this work we report on the purification as well as on the characterization of the structural, kinetic and regulatory properties of GAPN from celery leaves. Results are discussed in terms of the physiological relevance of GAPN in plants accumulating alditols.

2. Materials and methods

2.1. Materials

Fresh celery (*Apium graveolens* var. golden boy) leaves were obtained from plants grown in the field. DEAE-Sepharose fast flow, Mono Q HR5/5 and Superose 12 were from Pharmacia, Sweden. All other reagents were of the highest purity available and mainly obtained from either Sigma Chemical (St. Louis, MO, USA) or ICN (Argentina).

2.2. Enzyme assay

GAPN activity was determined spectrophotometrically at 30°C by monitoring NADPH generation at 340 nm in a HITACHI U-2000 recording spectrophotometer. The standard assay medium contained (unless otherwise specified) 50 mM Tricine–NaOH buffer (pH 8.5), 0.11 mM $NADP^+$, 1.2 mM D-Ga3P and an adequate quantity of enzyme in a total volume of 1 ml. Unless otherwise specified, after 2 min preincubation the reaction was started by addition of D-Ga3P, thus avoiding non-linear reaction rates produced by the hysteretic behavior exhibited by the enzyme under certain conditions (see Section 3). Alternatively, the reaction mixture contained 1.2 mM fructose-1,6-bisphosphate and 0.5 U of aldolase (from rabbit muscle), instead of D-Ga3P. The latter alternative was utilized to determine GAPN activity throughout purification. One unit (U) is defined as the amount of enzyme that catalyzes the formation of 1 μmol NADPH per minute under the specified assay conditions.

2.3. Protein measurements

Total protein was determined by the modified Lowry method [22]. BSA was used as standard.

2.4. Kinetic studies

All kinetic studies were performed using D-Ga3P as substrate. K_m values and Hill coefficients (n_H) for $NADP^+$ and D-Ga3P were determined using saturating concentrations of the respective non varied substrate. The experimental data were fitted to the Michaelis–Menten equation by a non-linear least-square regression kinetics computer program [23]. All kinetic parameters are the mean of at least two determinations and are reproducible to within $\pm 10\%$.

2.5. Purification of GAPN

All steps were performed at 5–10°C. Celery leaves (240 g) were washed, frozen under liquid N_2 and ground to a powder. The powdered material was extracted in a blender with a buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 40 mM 2-mercaptoethanol, 2 μM phenylmethylsulfonyl fluoride, filtered through two layers of Miracloth and then centrifuged at $12\,000 \times g$ for 15 min. The supernatant (crude extract) was brought to 35% saturation with crystalline ammonium sulfate and then centrifuged at $12\,000 \times g$ for 15 min. The

pellet was discarded and the supernatant adjusted to 70% saturation of the same salt and centrifuged. The precipitate containing GAPN was resuspended in a minimum volume of *buffer A* (25 mM Tris–HCl, 0.1 mM EDTA and 20 mM 2-mercaptoethanol, pH 7.5) and dialyzed against 400-volume of *buffer A* during 15 h.

The dialyzed sample was filtered through a 0.2 μm filter (Gelman Sciences). The clear supernatant was applied to a column (2.5 × 13 cm) of DEAE-Sepharose fast flow, previously equilibrated with *buffer A*. After exhaustive washing with the same buffer, the enzyme was eluted with a linear gradient consisting of 4-bed volumes of *buffer A* in the mixing chamber and 4-bed volumes of *buffer B* (*buffer A* supplemented with 400 mM KCl) in the reservoir chamber. Fractions with high GAPN activity were pooled, brought to 70% saturation with ammonium sulfate and centrifuged at 12 000 × *g* for 15 min. The precipitate was redissolved in *buffer A* and then dialyzed and filtered as above.

The sample was adsorbed onto a Reactive Red-120 agarose column (2.3 × 5 cm) preequilibrated with *buffer A*. After washing with 40 ml of the same buffer, the enzyme was eluted with a linear gradient of 0–400 mM KCl in *buffer A*. The pooled fractions were precipitated with 70% ammonium sulfate, redissolved, dialyzed and filtered as above. The sample was further concentrated to 0.2 ml with Ultrafree-MC (Millipore, NMWL 30 000) filters and then chromatographed on a Superose 12 HR10/30 column (flow rate 0.7 ml/min), using *buffer A* supplemented with 100 mM KCl. The active fractions were pooled, concentrated and dialyzed against *buffer A* with the Ultrafree-MC concentrator and applied to a Mono Q HR5/5 column. The enzyme was eluted from the ion exchange column with a linear gradient (10-bed volumes) using *buffer A* and *buffer B* as starting and final condition, respectively.

2.6. Protein electrophoresis, electroelution and immunoblotting

Non-denaturing PAGE was performed in 7.5% gels and runned at 4°C [24]. Gels were run at 150 V for 75 min. Total proteins in the gel were revealed using silver stain. GAPN was located in the gels by measuring activity and assaying the appearance of NADPH fluorescence under ultraviolet light (Fotodyne). The reaction was carried out by soaking the gel in a dish containing 10 ml of the standard assay medium for GAPN. After 5 min, the gel was placed on the ultraviolet lamp and the NADPH production was visualized as a yellow-green fluorescent band. A control was ran in parallel where the gel lane was soaked in similar conditions except for the absence of Ga3P in the medium.

Electrophoresis under denaturing conditions (SDS-PAGE) was performed in 9% gels running at 200 V for 40 min [25]. Following electrophoresis, gels were either, stained for protein or electroblotted onto nitrocellulose membranes using an Electrophoretic Transfer Cell (Bio Rad). After electroblotting, nitrocellulose membranes were treated with rabbit antiserum raised against denatured celery leaf GAPN and the antigen–antibody complex was visualized with alkaline phosphatase linked antirabbit IgG followed by staining with BCIP and NBT [22].

2.7. Molecular mass determination

The molecular mass of native GAPN from *Apium graveolens* was determined by using a Superose 12 HR10/30 column (FPLC-Pharmacia) calibrated with standard protein markers. The elution peak of GAPN was followed by measuring absorbance at 280 nm and checking for enzyme activity. Native molecular mass was determined from a plot of log molecular mass versus K_{AV} (partition coefficient) using the following proteins as standards: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa) and BSA (66 kDa). Void volume was determined using a solution of blue dextran. Subunit analysis was performed by SDS-PAGE using molecular mass markers from Sigma Chemical.

3. Results

3.1. Purification and molecular mass estimation of GAPN from celery leaves

The activity of GAPN in crude extracts from celery leaves was about 1.2 U/g fresh weight, with a specific activity (0.03 U/mg of protein); that is between two- and sixfold higher than values found

in other plant species [5,11]. These data are in agreement with a previous report by Rumpho et al. [20], indicating high levels of the non-phosphorylating enzyme in polyol producing plants. Purification of GAPN from celery leaves is shown in Table 1. The enzyme was purified about 1200-fold, with a recovery of 38% using a combination of ammonium sulfate fractionation; anion exchange, affinity and exclusion chromatography (Table 1). The procedure is reproducible and renders about 1.1 mg of purified enzyme per 100 g of celery leaves. The final specific activity (35 U/mg) was similar to those reported for GAPN from higher plants [12,19], green algae [11], and bacteria [26].

Fig. 1 shows the analysis of GAPN purified from celery leaves by PAGE run either under native or denaturing conditions. The results indicated that the purification procedure resulted in a pure enzyme according to this criterion. The purified enzyme migrated in native PAGE as a single major band revealed by silver staining the gel (Fig. 1A). This band exhibited GAPN activity, as it fluoresced, due to the presence of NADPH, after incubating the gel with Ga3P and $NADP^+$ (Fig. 1B). This clearly differenced respect to a control incubated under the same conditions except for the omission of Ga3P (Fig. 1C). In SDS-PAGE the purified enzyme ran as a single protein band of molecular mass 54 kDa (Fig. 1D). On the other hand, a molecular mass of 220 kDa was estimated for the native enzyme using gel filtration chromatography on a Superose 12 column (data not shown).

Antiserum raised in rabbits against celery leaf GAPN was tested for its capacity to neutralize enzyme activity. Increasing amounts of antiserum inhibited GAPN activity to near 100%, with the amount of antiserum necessary to cause 50% inhi-

Table 1
Purification of GAPN from 240 g of celery leaves

Fraction	Protein (mg)	Total act. (units)	Specific act. (units/mg)	Purification (×-fold)	Yield (%)
Crude extract	9586	278	0.029	1	100
Ammonium sulfate (35–70% saturation)	3958	285	0.072	2.5	103
DEAE-Sepharose	400	210	0.53	18	76
Reactive red	20	206	10.3	355	74
Superose 12	8	178	22.3	769	64
Mono Q	3	105	35.0	1207	38

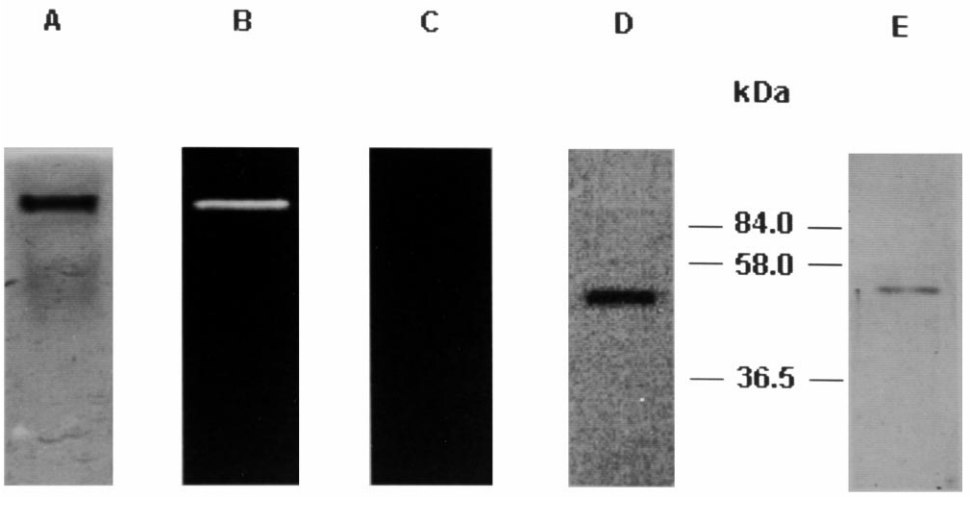

Fig. 1. PAGE of GAPN purified from celery leaves. (A) Silver stained native PAGE of the pure enzyme (0.5 μg). (B) Native PAGE revealed by GAPN activity (0.8 μg). (C) Control of activity staining by incubation of the lane as in B but in the absence of Ga3P. (D) Silver stained SDS-PAGE of the purified enzyme (0.5 μg). (E) Immunoblot analysis of the SDS-PAGE ran for the purified enzyme (0.5 μg).

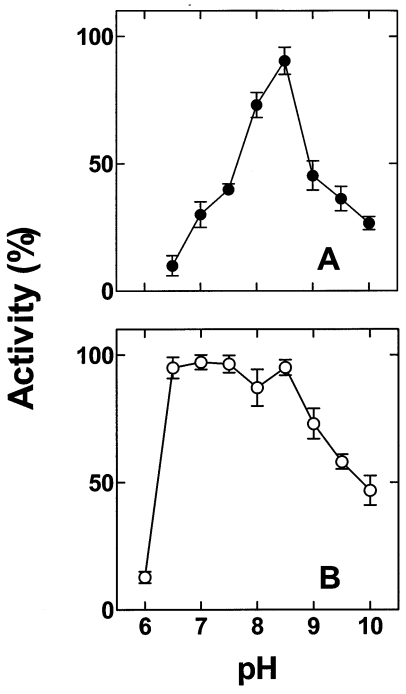

Fig. 2. Activity and stability of GAPN from celery leaves at different pH values. The enzyme was assayed for activity (A) at different pHs or preincubated (B) at the specified pH values and then assayed at pH 8.5. Hundred percent of activity corresponds to the value obtained at pH 8.5 (35 units/mg of protein).

Table 2
Kinetic parameters for substrates and products of GAPN purified from celery leaves

Compound	K_m (μM)	n_H	K_i (μM)	Type of inhibition
$NADP^+$	7 ± 0.5	1.1 ± 0.1		
D-Ga3P	127 ± 10	1.0 ± 0.1		
L-Ga3P			30 ± 2	NC[a], respect to D-Ga3P
NADPH			12 ± 1	C, respect to $NADP^+$
			10 ± 1	C, respect to D-Ga3P
3PGA			525 ± 33	C, respect to D-Ga3P
			254 ± 20	C, respect to $NADP^+$

[a] NC, non-competitive inhibitor; C, competitive inhibitor.

bition ($I_{0.5}$) being 43 μl per mU of enzyme (data not shown). This antiserum was effective when used for immunoblot analysis of SDS-PAGE, as shown in Fig. 1E.

3.2. Kinetic properties of the enzyme

GAPN purified from celery leaves catalyzed the irreversible oxidation of Ga3P utilizing specifically $NADP^+$ (no activity was observed when NAD^+ was used as a substrate). Fig. 2 shows the activity of the enzyme assayed or incubated at different pH values. Maximal activity was observed when the enzyme was assayed at pH 8.5 (Fig. 2A). The enzyme was stable to 5 min incubation in the pH range 6.5–8.5, whereas low stability was observed above pH 8.5 and was markedly reduced below pH 6.5 (Fig. 2B). At pH 8.5 saturation curves of the enzyme for the substrates followed hyperbolic kinetics with K_m values of 7 and 127 μM for $NADP^+$ and Ga3P, respectively (Table 2).

GAPN exhibited a high specificity to use D-Ga3P with the L-isomer behaving as a potent inhibitor. From experiments carried out by assaying the enzyme activity at different DL-Ga3P:D-Ga3P ratios (obtained by mixing different amounts of the DL- and D-forms), it was determined that L-Ga3P inhibited the enzyme in a non-competitive manner, with a K_i value of 30 μM (Table 2). Concerning the products of the reaction, both 3PGA and NADPH behaved as competitive inhibitors with respect to both of the substrates, and the corresponding K_i values are shown in Table 2. The enzyme activity was not affected by addition (up to 1.5 mM concentration) of Pi or arsenate in the assay medium (data not shown). On the other hand, the enzyme was inhibited by increasing concentrations of KCl, with 50% inhibition reached at 160 mM salt (data not shown). This effect seems to be caused by ionic strength, since similar results were observed with other salts.

3.3. Stability and hysteretic behavior of GAPN

It has been reported that GAPN from plants requires the maintenance of essential sulfhydryl residues in the reduced state to remain active [11]. In our hands, this condition was necessary but not sufficient to stabilize the enzyme purified from celery leaves. Thus, the enzyme activity decreased to about 20% of the initial value within 1 week when stored in *buffer A* (which contains 2-mercaptoethanol) at 4°C (similar results were obtained at −20°C). The storage of an active enzyme also required conditions of high ionic strength (0.5 M KCl or 60% saturation ammonium sulfate) or the presence of 10 mM $NADP^+$ in *buffer A*. Under

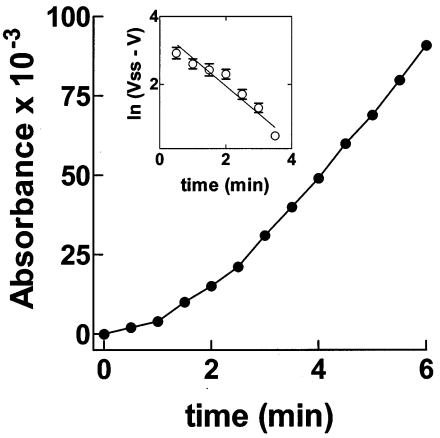

Fig. 3. Time course of the reaction of GAPN from celery leaves. The assay was performed at pH 8.5 as specified in the text and started by the addition of the enzyme preincubated under the same conditions but in the presence of substrates. Production of NADPH was followed spectrophotometrically at 340 nm. Inset: fitting of the main Figure to the equation developed by Neet and Ainslie (28) for enzymes exhibiting hysteretic behavior.

Table 3
Effect of different compounds on the hysteretic properties exhibited by GAPN purified from celery leaves[a]

Addition	τ (min)	V_i (units/mg)	V_{ss}
None	2.8 ± 0.2	7.5 ± 0.7	35 ± 3
D-Ga3P (1 mM)	1.2 ± 0.1	15 ± 1	35 ± 3
$NADP^+$ (0.1 mM)	0.3 ± 0.03	30 ± 3	45 ± 4
(1 mM)		66 ± 6	66 ± 6
3PGA (1 mM)	0.6 ± 0.04	7.5 ± 0.4	30 ± 2
NADPH (1 mM)	0.4 ± 0.04	10 ± 1	30 ± 3
NAD^+ (1 mM)	2.5 ± 0.2	8.0 ± 0.1	32 ± 3
NADH (1 mM)	2.7 ± 0.2	8.5 ± 0.1	29 ± 3
KCl (1 M)	0.9 ± 0.1	11 ± 0.1	22 ± 2
Polyethylenglycol (20%, w/v)	2.1 ± 0.2	4.5 ± 0.2	22 ± 2
BSA (25 mg/ml)	1.9 ± 0.1	4.0 ± 0.3	25 ± 2
$MgCl_2$ (5 mM)	3.1 ± 0.3	6.0 ± 0.6	28 ± 3
$CaCl_2$ (5 mM)	3.0 ± 0.3	5.5 ± 0.5	27 ± 3

[a] Purified GAPN was preincubated at 30°C in a medium containing 50 mM Tricine–NaOH, pH 8.5, 10 mM 2-mercaptoethanol and the different specified compounds. After 2 min preincubation, an aliquot was withdrawn and immediately assayed for activity as described in Section 2. To assure that the effect of the different compounds is on the hysteretic behavior of the enzyme specifically, they were investigated at relevant concentrations in the assay medium. Thus, controls showing no effect on catalysis of the amount of effector introduced from the preincubation mixture into the assay medium were carried out in parallel.

any of the latter conditions, about 80% of activity was retained after 1 month storage at 4°C.

Fig. 3 shows a time course of the reaction catalyzed by GAPN purified from celery leaves. As shown, changes in absorbance at 340 nm revealed the occurrence of non-linear reaction rates when assay for activity was started by the addition of enzyme previously incubated under similar conditions except in the absence of substrates (Fig. 3). Under these conditions, the enzyme activity reached maximal values after a lag period, which was shortened or eliminated, respectively, by the presence of Ga3P (1 mM) or $NADP^+$ (1 mM) in the preincubation medium (see Table 3). These transient kinetics exhibited by GAPN point out a hysteretic behavior of the enzyme, which is modified by the substrates at rates slower than the rate of catalysis [27,28]. The kinetic transient in Fig. 3 followed a first-order exponential increase, since data can be fitted (as shown in the inset of Fig. 3) to the equation described by Neet and Ainslie [28]:

$$\ln(V_{ss} - V) = \ln(V_{ss} - V_i) - t/\tau \quad (1)$$

where V, V_i and V_{ss} are the instantaneous, the initial and the steady state (linear) velocities, respectively; t is time of the reaction and τ is the inverse of the apparent rate constant for the transition between V_i and V_{ss}.

The presence of substrates, products or different metabolites in the preincubation medium affected the hysteretic behavior of GAPN. Table 3 shows values for V_i, V_{ss} and τ calculated by using Eq. (1) from time courses of activity exhibited by the enzyme incubated in the presence of different compounds. As can be seen, in the absence of additions enzyme activity reached a V_{ss} value fivefold higher than V_i with a lag period of minutes ($\tau =$ 2.8 min). Incubation of GAPN with Ga3P decreased the τ value whereas that the presence of 1 mM $NADP^+$ in the incubation medium abolished the hysteretic behavior and also induced an activation of the enzyme, thus reaching a V_{ss} nearly twofold higher; whereas at 0.1 mM $NADP^+$ highly reduced τ and slightly increased the velocity value (Table 3). On the other hand, the products of the reaction catalyzed by GAPN markedly modified the hysteretic behavior by reducing the lag period (Table 3). As also shown in Table 3, the effect of $NADP^+$ and NADPH was selective, since no effect was observed with NAD^+ or NADH.

Table 4
Effect of mannitol, sorbitol, sucrose and glycerol on the hysteretic properties exhibited by GAPN purified from celery leaves[a]

Addition	τ (min)	V_i (units/mg)	V_{ss}
None	2.8 ± 0.2	7.5 ± 0.7	35 ± 3
Mannitol (M)			
0.005	2.8 ± 0.2	7.5 ± 0.7	35 ± 3
0.05	2.7 ± 0.2	7.5 ± 0.7	38 ± 4
0.25	2.1 ± 0.2	8.0 ± 0.7	42 ± 3
0.5	1.4 ± 0.1	8.5 ± 0.8	47 ± 4
1	1.1 ± 0.1	8.5 ± 0.8	49 ± 4
Sorbitol (M)			
0.25	2.5 ± 0.2	8.0 ± 0.7	40 ± 3
1	1.4 ± 0.1	7.0 ± 0.7	47 ± 5
Sucrose (M)			
0.25	2.4 ± 0.2	8.0 ± 0.7	36 ± 3
1	1.6 ± 0.1	10 ± 1	40 ± 4
Glycerol (M)			
0.25	2.4 ± 0.2	7.5 ± 0.6	37 ± 3
1	1.5 ± 0.1	6.0 ± 0.5	37 ± 3

[a] Preincubation conditions, assay for activity and controls, were carried out as specified in Table 3.

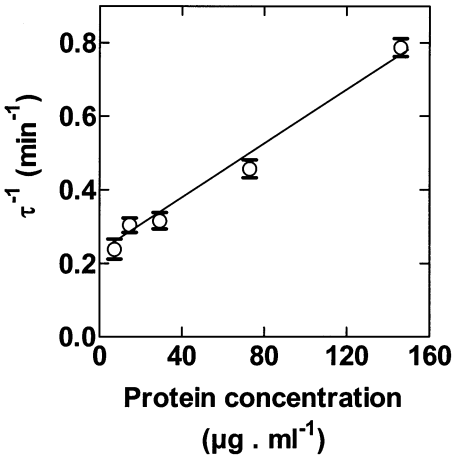

Fig. 4. Effect of protein concentration in the preincubation medium on the lag phase of the hysteretic behavior exhibited by GAPN. The enzyme was preincubated at pH 8.5 at different protein concentrations and then assayed for activity at pH 8.5.

The presence of KCl (1M) in the incubation medium reduced τ value and V_{ss} but showed no effect on V_i whereas PEG and BSA caused a reduction in the three parameters (Table 3). The effect of divalent metal ions (Mg^{2+} and Ca^{2+}) on the hysteretic behavior of GAPN is also illustrated in Table 3, these cations slightly increased the lag and reducing velocity values.

The hysteretic behavior exhibited by GAPN was also affected by mannitol. Table 4 shows that mannitol modified, in a concentration dependent manner, hysteretic parameters of the enzyme, by shortening the lag period and increasing V_i and V_{ss} by 1.1- and 1.4-fold, respectively. Sorbitol exhibited a similar effect to that afforded by mannitol, whereas sucrose and glycerol only affected τ, reducing its value by nearly 50%, but having no effect on velocity values (Table 4). The effect of mannitol may be of physiological relevance, since in celery this compound accumulates, being a major product of the photosynthetic process [20,21].

The transient lag exhibited by GAPN was dependent on the protein concentration in the preincubation medium. Fig. 4 illustrates that the lag time was reciprocally related to the enzyme concentration, as expected from a system in which hysteretic behavior results from changes in the aggregation state of the enzyme. However, the plot does not originate from zero as would be expected for an infinite lag at extremely low protein concentrations; thus suggesting the occurrence of a conformational change in the protein in addition to the aggregation effect.

4. Discussion

The data reported here, on the presence of relatively high levels of GAPN activity in celery leaves agree with an early report of Rumpho et al. [20] showing the involvement of the enzyme in the photosynthetic metabolism of this mannitol producing plant. In the cited paper, Rumpho et al. describe a metabolic scenario where triose-P participates in the production of NADPH via GAPN in the cytosol and the product 3PGA is interexchanged through the Pi-translocator of the chloroplast envelope to regenerate triose-P necessary for carbon supply for the synthesis of mannitol (for details see [20]).

We purified GAPN from celery leaves to electrophoretic homogeneity, to reach a specific activity of 35 U/mg of protein. So far, this is the first purification and characterization of GAPN from a plant with an active metabolism for the accumulation of alditols. Gel filtration chromatography and SDS-PAGE studies showed a homotetrameric structure of the enzyme, with a native molecular mass of 220 kDa. These structural properties are similar to those previously found for GAPN from different sources (for a review see [6]).

Purified GAPN specifically utilized as substrates $NADP^+$ and the D-form of Ga3P. Interestingly, L-Ga3P was found to be a non-competitive inhibitor of the enzyme, in agreement with results reported for GAPN from green algae; and NAD^+ was not effective as a substrate nor as an inhibitor of the enzyme. The products of the reaction, NADPH (but not NADH) and 3PGA behaved as inhibitors, competitive with respect to both of the substrates, as previously shown for the enzyme from spinach leaves and green algae ([11,12]; see also Ref. [6]). Thus, although it was demonstrated that GAPN is not related to glyceraldehyde-3P dehydrogenases phosphorylating, but to aldehyde dehydrogenases [15]; a clear difference can be established, since the latter are relatively non-specific dehydrogenases and plant GAPN can be distinguished by their high specificity for the substrates.

GAPN from celery leaves exhibited a relatively high instability. Besides the necessity of the presence of a sulfhydryl reducing agent (2-mercaptoethanol) due to the presence of essential cysteinyl residues [26], the celery enzyme required conditions of high ionic strength or the presence of $NADP^+$ to remain stable for one month at 4°C. Moreover, the purified enzyme exhibited hysteretic properties, performing non-linear reaction rates with a lag in the activity observed when reaction was started with enzyme preincubated in the assay condition, but without substrates. Results suggest that the enzyme undergoes a transition to a more active form when exposed to substrates, and that the modification process occurs slower than the catalytic step. This proposed conformational change seems to be enhanced by $NADP^+$, which abolished the lag period and stimulated the enzyme activity by nearly twofold. Similar properties were previously reported for GAPN from spinach leaves [12]. It has been proposed that the activating effect of $NADP^+$ on plant GAPN could be relevant for the maintenance of a high cytosolic $NADPH/NADP^+$ ratio in vivo (see [12]). Thus, present results fit well in a metabolic picture where the enzyme plays a key role in the partitioning of redox equivalents in plant cells.

The actual significance of the hysteretic behavior of GAPN under in vivo conditions is not clear, since it is affected by substrates, protein concentration and level of different compounds (i.e. mannitol) determining a complex mixture in the cell. However, it is tempting to speculate that changes occurring in the level of carbon intermediates within photosynthetic cells could modify the enzyme activity in a distinctive and effective manner. In this way, the effect of mannitol may be particularly relevant, since the high amounts of this compound accumulate in celery [21].

The physiological role for GAPN in photosynthetic cells of higher plants was proposed by Kelly and Gibbs [17]. In this model, the function of the enzyme is to supply the cytosol with NADPH by oxydizing Ga3P produced in the Calvin cycle and transported from the plastid via the Pi-translocator (reviewed in [6]). Indeed, the enzyme is involved in a transport shuttle system that facilitates carbon (and also reducing equivalents after GAPN function) partitioning between chloroplast and cytosol, and has been well characterized in plants that synthesize sucrose and starch as the major final products of photosynthesis [17,6]. Plants synthesizing alditols require additional NADPH in the cytosol to actively produce sugar alcohols as major products of photosynthesis and compatible solutes for coping with stress conditions [21]. As a whole, the results suggest that the involvement of GAPN in the above cited transport shuttle system plays a key role in plants that accumulate large amounts of alditols. In this way, the enzyme provides reducing equivalents for the production of one main photosynthetic product and thus it is involved in the process of photoassimilates partitioning between starch, sucrose and alditols.

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