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ARTICLE *in* PLANT SCIENCE · MAY 2002

Impact Factor: 3.61 · DOI: 10.1016/S0168-9452(02)00015-8

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On the interaction of substrate analogues with non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase from celery leaves

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Received 5 September 2001; received in revised form 28 December 2001; accepted 2 January 2002

Abstract

Structural analogues of D-glyceraldehyde-3-phosphate (D-Ga3P) and $NADP^+$ were studied as potential substrates and inhibitors of the non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9; GAPN) from *Apium graveolens* leaves. Four analogues of $NADP^+$: nicotinamide-hypoxanthine dinucleotide phosphate ($NHDP^+$); 3-acetylpyridine-adenine dinucleotide phosphate ($APADP^+$); 1,N^6-etheno-nicotinamide dinucleotide phosphate ($\varepsilon NADP^+$); and β-nicotinamide adenine dinucleotide 2′:3′-cyclic monophosphate (2′3′$NADP^+$c) were alternative enzyme substrates, with a 2-fold variation in V_{max} and 2–11-fold differences in K_m compared to $NADP^+$. These compounds were also able to affect the hysteretic behavior of celery GAPN, in a similar way as the substrate $NADP^+$. The analogues to the nicotinamide moiety: thionicotinamide and 3-aminopyridine, behaved as competitive inhibitors, exhibiting a high-affinity binding to the enzyme. All the analogues of D-Ga3P that were analyzed behaved as competitive inhibitors, except for the L-isomer of the substrate which at relatively high concentrations exhibited a non-competitive effect. Results showed the importance of: (i) the chemical group at carbon-1 of Ga3P; (ii) the presence of a phosphate ester at carbon-3, and (iii) the stereochemical configuration at carbon-2. The particular behavior of L-Ga3P is analyzed by differences in tridimensional structure between bacteria and plant GAPNs.

Keywords: Non-phosphorylating; Glyceraldehyde-3-phosphate dehydrogenase; GAPN; Celery; $NADP^+$; Glyceraldehyde-3-P analogues

1. Introduction

The NADP-dependent, non-phosphorylating, glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:$NADP^+$ oxidoreductase; EC 1.2.1.9; abbrev. GAPN) catalyzes the essentially irreversible reaction: Ga3P + $NADP^+$ ⇒ 3P-glycerate + NADPH + 2 H^+. The enzyme was first described in photosynthetic cells by Arnon et al. [1,2] and then purified and characterized from a number of vascular plants [3–8] and green algae [9]. The enzyme was also reported in eubacteria [10] and archaebacteria [11]. A recent review [12], deals with the biochemical and structural properties of GAPN from different sources and details the involvement of the enzyme in central metabolic pathways.

Different physiological roles have been suggested for GAPN [12]. In vascular plants GAPN participates in a shuttle system for the export of photosynthetically-generated NADPH from the chloroplast to the cytosol [13,14]. In plants accumulating acyclic polyols (such as celery) GAPN seems to supply the NADPH necessary for the synthesis of these reduced sugars, thus playing a key role in photoassimilate partitioning [7,8]. In non-green plant tissues, GAPN may supply reducing power

Abbreviations: $AADP^+$, 3-aminopyridine-adenine dinucleotide phosphate; $APADP^+$, 3-acetylpyridine dinucleotide phosphate; Ga3P, glyceraldehyde-3-phosphate; $\varepsilon NADP^+$, 1,N^6-etheno-nicotinamide adenine dinucleotide phosphate; 2′3′$NADP^+$c, β-nicotinamide adenine dinucleotide 2′:3′-cyclic monophosphate; 3′$NADP^+$, β-nicotinamide adenine dinucleotide 3v phosphate; $NHDP^+$, nicotinamide-hypoxanthine dinucleotide phosphate; $SNADP^+$, thionicotinamide-adenine dinucleotide phosphate.

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0168-9452/02/$ - see front matter
PII: S0168-9452(02)00015-8

for anabolic reactions coupled with glycolysis [12]. In bacteria, the enzyme could mainly function as an NADPH producer, due to the lack of the oxidative pentose phosphate pathway and transhydrogenase activity in these organisms [12].

GAPN belongs to the aldehyde dehydrogenase superfamily as indicated by the stereospecificity of the cofactor reduction [15], and comparative sequence analysis of cloned proteins [16]. In agreement with this, GAPN catalysis occurs via the thioester pathway, a common feature of aldehyde dehydrogenases [12]. The aldehydic group of the substrate (Ga3P) is converted into a tetrahedral intermediate by forming a thiohemiacetal with a specfic sulfhydryl residue in the protein, a necessary step for the transfer of a hydride ion to the C-4 position of the nicotinamide ring of $NADP^+$ [6,17,18].

It has been reported that binding of $NADP^+$ to GAPN from *Streptococcus mutants* induces a conformational rearrangement that increases the reactivity of the essential cysteine-284 [17]. Moreover, the crystal structure of apo- and holo-GAPN from *S. mutants* was recently solved [19]. From these studies, relevant information was obtained on the structure of substrate binding sites and on the role played by specific amino acid residues in the protein [20]. Further studies are necessary to confirm the structural models thus proposed for the bacterial enzyme as well as to establish possible similarities and differences with GAPN from plants.

In the present work we used kinetic analysis to characterize the specificity for substrates of GAPN purified from celery leaves. By using different chemical analogues of $NADP^+$ and Ga3P we obtained information on geometrical parameters and charge distribution that govern the interaction of different compounds with the enzyme. Results are analyzed comparatively with others on the structure of the crystallyzed bacterial enzyme as an approach in the characterization of the structural properties of plant GAPN.

2. Materials and methods

2.1. Chemicals

$NADP^+$, $NHDP^+$, $APADP^+$, $\varepsilon NADP^+$, 2′3′$NADP^+$c, $SNADP^+$, $AADP^+$, 3′$NADP^+$, DL-α-glycerophosphate, L-α-glycerophosphate, β-glycerophosphate, D-glyceraldehyde-3-phosphate, DL-glyceraldehyde-3-phosphate, DL-glyceric acid, and D-3P-glycerate were purchased from Sigma (St. Louis, MO). All other chemicals were of reagent grade.

2.2. Protein measurement

Total protein was determined by the modified Lowry method [21]. Bovine serum albumin was used as standard.

2.3. Enzyme purification and assay

GAPN from celery (*Apium graveolens* var. Golden Boy) leaves was purified according to a procedure described previously [7]. Enzyme activity was determined spectrophotometrically at 30 °C by monitoring NADPH production 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 GAPN purified from celery leaves in a final volume of 1 ml. To avoid non-linear reaction rates produced by the hysteretic behavior reported for GAPN from celery [7], the purified enzyme was preincubated for 3 min in the assay medium and then the reaction was started by addition of D-Ga3P. Alternatively (when possible), the reaction mixture contained 2.4 mM fructose-1,6-bisphosphate and 0.5 U of aldolase (from rabbit muscle), instead of D-Ga3P. One unit (U) is defined as the amount of enzyme that catalyzes the formation of 1 μmol NADPH/min under the specified assay conditions.

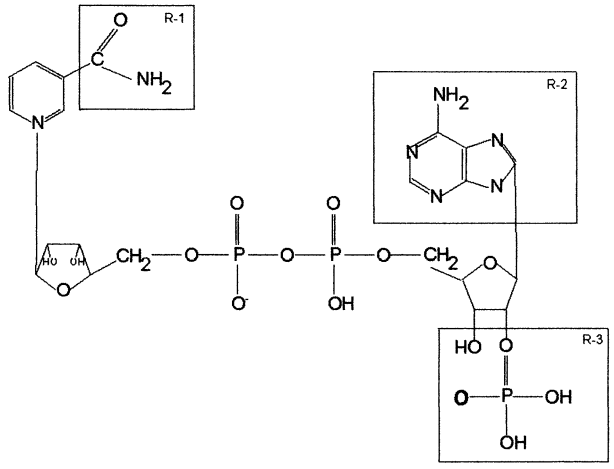
2.4. Kinetic studies

Initial velocity studies were performed by varying concentrations of $NADP^+$ (or the respective analogue) from 0.001 to 0.5 mM and D-Ga3P from 0.01 to 5 mM. $NADP^+$ was fixed at 0.11 mM and D-Ga3P was maintained at 1.2 mM when the respective cosubstrate was varied. Inhibition studies were performed in a similar manner by varying the concentration of $NADP^+$ or D-Ga3P while the corresponding analogue (behaving as inhibitor) was fixed at several different concentrations around the respective inhibition constant.

The experimental data were fitted to the generalized Hill equation by a non-linear least square regression kinetics computer program [22]. This program was used to determine sigmoidicity in the respective substrate saturation curve, by calculating Hill coefficients (n_H). Since n_H was not significantly different from 1 in all cases, the same computer program was utilized to fit data to the Michelis–Menten equation.

Initial velocity data exhibiting linear competitive or non-competitive inhibition were fitted to Eqs. (1) and (2), respectively (see Ref. [23]):

$$v = \frac{V_{max} \cdot [S]}{K_m(1 + [I]/K_i) + [S]} \quad (1)$$



Compound	R-1	R-2		R-3
$NADP^+$	nicotinamide	adenine	dinucleotide	2' phosphate
$APADP^+$	**3-acetylpyridine**	adenine	dinucleotide	2'phosphate
$AADP^+$	**aminopyridine**	adenine	dinucleotide	2'phosphate
$SNADP^+$	**thionicotinamide**	adenine	dinucleotide	2'phosphate
$\varepsilon NADP^+$	nicotinamide	**ethenoadenine**	dinucleotide	2'phosphate
$NHDP^+$	nicotinamide	**hypoxanthine**	dinucleotide	2'phosphate
$2',3'NADPc^+$	nicotinamide	adenine	dinucleotide	**2'3'-cyclic monophosphate**
NAD^+	nicotinamide	adenine	dinucleotide	**2'-OH**
$3'NADP^+$	nicotinamide	adenine	dinculeotide	**3' phosphate**

Fig. 1. Structure of $NADP^+$ and the analogues with chemical variations in one of the three regions of the pyridine nucleotide molecule.

$$v = \frac{V_{max} \cdot [S]}{(K_m + [S]) \cdot (1 + [I]/K_i)} \quad (2)$$

where [S] is the concentration of $NADP^+$ (or the analogue exhibiting activity) or D-Ga3P, [I] is the concentration of the inhibitor, and K_i is the apparent inhibition constant.

All kinetic parameters are means of at least three determinations and are reproducible within at least $\pm$ 10%.

3. Results and discussion

3.1. Analogues of $NADP^+$ as substrates or inhibitors

To analyze the role of different parts of the $NADP^+$ molecule in the formation of a functionally competent complex with GAPN, several analogues of the nicotinamide nucleotide were proved as substrates or inhibitors of the enzyme purified from celery leaves. Compounds tested were analogues with variations in one of the three regions of the pyridine nucleotide molecule (Fig. 1). As shown, region 1 (R-1) corresponds to the amide group in the nicotinamide ring, region 2 (R-2) is the adenine moiety, and region 3 (R-3) the ribose structure of the entire molecule. Table 1 summarizes the kinetic results obtained with $NADP^+$ and the eight analogues analyzed.

Analogues with variations in R-2 were somewhat active as alternative substrates of GAPN, suggesting that an intact adenine structure is not absolutely essential for catalysis. Activities with $NHDP^+$ and $\varepsilon NADP^+$ exhibited apparent V_{max} values similar to that of the natural substrate, although K_m values determined for both analogues were between 5- and 6-fold higher than for $NADP^+$; this resulted in a lower specificity constant, represented by the ratio V_{max}/K_m in Table 1 [24]. All this suggests that the adenine moiety is relevant for correct binding to the enzyme. The other analogues acting as substrates of the enzyme were $APADP^+$, a compound modified in R-1; and the R-3 cyclic phosphate derivative $2',3'NADPc^+$ (Table 1). $APADP^+$ exhibited similar catalytic efficiency than the analogues in R-2, as a result of an about 2-fold increase in K_m and decrease in V_{max} relative to kinetic data for

Table 1
Kinetic parameters or inhibition constants for $NADP^+$ and several analogues acting as substrates or inhibitors of GAPN from celery leaves

Compound	K_m (μM)	V_{max} (U/mg)	V_{max}/K_m ($\mu M^{-1}/s$)	K_i (μM)	Type of effector[a]
$NADP^+$	7.0	35.0	16.7		S
$NHDP^+$	33	37.1	3.73		S
$APADP^+$	16	16.8	3.50		S
$\varepsilon NADP^+$	40	32.2	2.70		S
$2',3'NADPc^+$	80	21.4	0.90		S
$SNADP^+$				0.34	Ci
$AADP^+$				0.45	Ci
NAD^+					n.e.[b]
$3'NADP^+$					n.e.

[a] S, substrate; Ci, competitive inhibitor respect to $NADP^+$.
[b] n.e., no effect was observed when tested up to 2 mM.

Table 2
Effect of $NADP^+$ and its analogues on the hysteretic properties exhibited by GAPN purified from celery leaves[a]

Compound	τ[b] (min)	V_i (U/ml)	V_{ss}
None	2.9	1.2	5.5
$NADP^+$		10.1	
$NHDP^+$		10.9	10.9
$APADP^+$		6.3	6.3
$\varepsilon NADP^+$		9.9	9.9
$2',3'NADPc^+$	0.3	3.5	7.0
$SNADP^+$	2.7	1.0	4.9
$AADP^+$	3.3	1.1	5.0
NAD^+	2.5	1.4	5.5
$3'NADP^+$	2.7	1.2	5.4

[a] Studies on the hysteretic behavior were performed as previously described [7]. The purified enzyme 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 at 1 mM concentration. After 3 min preincubation, an aliquot was withdrawn and immediately assayed for activity. To assure that the effect of the different compounds was on the hysteretic behavior of the enzyme, controls showing no effect on catalysis of the amount of effector introduced from the preincubation into the assay medium were carried out in parallel.
[b] Abbreviations: τ is the inverse of the apparent rate constant for the transition between the initial (V_i) and the steady state (linear, V_{ss}) velocities (see also Ref. [7]).

$NADP^+$; whereas $2',3'NADPc^+$ exhibited a very poor specificity constant, mostly because the high apparent K_m for this compound (Table 1).

The analogues in the amide group of the nicotinamide region, $SNADP^+$ and $AADP^+$, behaved as competitive inhibitors of $NADP^+$ (Table 1). When analyzed, K_i values for these analogues are markedly lower than K_m values for those compounds that are enzyme substrates, thus indicating a relative high affinity. Results obtained with $SNADP^+$ and $AADP^+$ show the relevance of the carbonyl group of the nicotinamide carboxamide in the hydride transfer reaction. Molecular modeling derived from X-ray crystallography for GAPN from *S. mutants* indicates that the carboxamide group of nicotinamide is maintained by a hydrogen bond with the main chain carbonyl group of a Leu residue in the protein [19,20]. Modifications in the analogues ($SNADP^+$ and $AADP^+$) affecting the hydrogen binding ability seem to modify the orientation of the nicotinamide ring, making it unable to participate in catalysis.

On the other hand, Table 1 shows that the presence of the phosphate group in the ribose moiety of the substrate is critical not only for activity but also for the binding to GAPN; since NAD^+ had no effect on enzyme activity. This agrees with the high specificity for $NADP^+$ reported for several plant and bacteria GAPNs [12]. Cobessi et al. [19,20], after analysis of X-ray crystallography data, concluded that a Thr residue located in a steric constraint in GAPN interacting with the phosphate group of $NADP^+$ determined specificity. Results here, with the cyclic phosphate derivative $2',3'NADPc^+$ as an alternative substrate, suggest that the analogue also interacts with the Thr residue; although the structural modification determines a lower affinity for binding. Remarkably, when the phosphate group is at position 3′ ($3'NADP^+$, see Table 2) no interaction seems to occur between the analogue and GAPN, suggesting a very strict steric limitation for the establishment of the interaction.

It has been reported that celery GAPN exhibits a hysteretic behavior, with a lag phase of minutes, when activity is assayed with enzyme preincubated in the absence of substrates [7]. The presence of $NADP^+$ in the preincubation medium mainly affects the hysteretic behavior as 1 mM of the substrate abolishes the lag period and produces a near 2-fold activation of the enzyme [7]. Table 2 shows the effect of the different analogues of $NADP^+$ on the parameters (τ, V_i, and V_{ss}; see abbreviations in Table 2) characterizing the hysteretic properties of GAPN. As shown, not only $NADP^+$, but also $NHDP^+$, $APADP^+$, and $\varepsilon NADP^+$ were effective in eliminating the hysteretic behavior and activating the enzyme activity between 1.15- and 1.98-fold. A similar effect was produced by $2',3'NADPc^+$ which shortened the lag period from 2.9 to 0.3 min and increased V_{ss} by 1.3-fold; whereas the remaining analo-

Table 3
Inhibition constants and pattern for the inhibition of celery leaves GAPN by several D-Ga3P analogues

Compound	K_i (μM)	Inhibition pattern[a]
L-Ga3P	5.5	Ci (< 40 μM)
	37	NCi (> 75 μM)
β-glycerophosphate	339	Ci
DL-α-glycerophosphate	450	Ci
L-α-glycerophosphate	940	Ci
DL-glyceraldehyde	1050	Ci
D-3P-glycerate	525	Ci
DL-glyceric acid		n.e.[b]

[a] Ci, competitive inhibitor; and NCi, non-competitive inhibitor respect to D-Ga3P.
[b] n.e.: no inhibitory effect was observed when tested up to 2 mM.

gues showed little or no effect on the hysteretic properties of the enzyme (Table 2).

Interestingly, results in Table 2 show that compounds affecting the hysteretic behavior of GAPN are those that are active as substrates of the enzyme; with the capability to reduce or eliminate the lag period and in to increase V_{ss} being somehow related with the specificity constant exhibited by the alternative substrate (see data in Table 2 compared with those in Table 1). Since the hysteretic behavior of GAPN supposes a mechanism for regulation of the enzyme activity [7], the present results suggest that the possible occurrence of the hysteretic behavior in vivo could be modify by compounds that affect not only the binding of $NADP^+$ to the enzyme, but also the dynamic use of the nucleotide as a substrate in the catalytic reaction.

3.2. D-Ga3P analogues as inhibitors.

GAPN from different plants (including the celery leaf enzyme) has been characterized as exhibiting a very high specificity to oxidize D-Ga3P [6–9,12]. We analyzed the effect of different three-carbon analogues of D-Ga3P to determine how changes in the chemical and stereochemical structure at carbons 1, 2, or 3 of the substrate affect the kinetic properties of the enzyme purified from celery leaves. Table 3 shows the effect on enzyme activity and kinetics produced by seven different analogues of D-Ga3P.

Analogues where the C-1 of Ga3P is modified by replacing the aldehyde group by an alcohol behaved as competitive inhibitors with the relative affinity dependent on the stereochemistry at C-2 (Table 3). Thus, the L-form of α-glycerophosphate exhibited a lower affinity than the mixture of DL-forms as evident from a K_i value about 2 times higher. On the other hand, β-glycerophosphate was more effective as inhibitor, with a K_i value of 339 μM (Table 3), showing that a compound with a phosphate group at C-2 (instead of C-3) is also able to interact with the enzyme. When the aldehyde group at

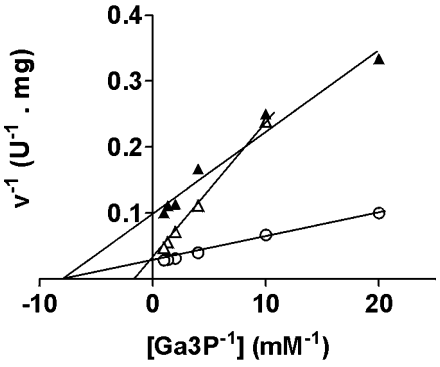


Fig. 2. Double reciprocal plot of GAPN activity vs. D-Ga3P concentration. Assays were performed as indicated under Section 2 in the absence (○) or in the presence of 30 μM (△) or 100 μM (▲) L-Ga3P.

C-1 was replaced by a carboxylic acid, the resulting analogue inhibited GAPN if the phosphate group at C-3 was still present. Thus, 3P-glycerate was a competitive inhibitor with a K_i of 525 μM, but glyceric acid had no effect on the enzyme activity (Table 3). The critical role of the phosphate group at C-3 is shown by the effect of glyceraldehyde, which was ineffective as an enzyme substrate and a relatively poor competitive inhibitor, with a K_i of about 1.1 mM (Table 3).

A particular kinetic effect was observed in relation to the stereochemical configuration at C-2 of Ga3P. As previously reported [7] GAPN purified from celery leaf exhibits a V_{max} of 35 U/mg with a K_m for D-Ga3P of 127 μM. The L-form of Ga3P is not effective as a substrate, but behaves as an inhibitor of the enzyme, which underscores the relevance of the stereochemistry at C-2 of Ga3P (Table 3). This was previously analyzed as a characteristic of GAPNs from plants and green algae, with L-Ga3P reported as a potent non-competivive inhibitor of these enzymes [12].

We performed a more detailed analysis of the effect of L-Ga3P on GAPN from celery leaf. Our results show that the type of L-Ga3P inhibition is different when studied at concentrations lower than 40 or higher than 75 μM (Table 3). Fig. 2 shows that 30 μM L-Ga3P inhibited the celery enzyme in a competitive manner respect to D-Ga3P, with a K_i of 5.5 μM; whereas at 100 μM the inhibition was non-competitive with a K_i of 37 μM (see also Table 3).

An explanation for this particular inhibitory effect of the stereochemical analogue of the substrate is that the aldehydic group of L-Ga3P forms a thiohemiacetal with a specific sulfhydryl residue in the enzyme (as occurs with D-Ga3P, see [6,17]). In fact, results show a very high affinity of the L-isomer for the binding to the enzyme, as deduced from the low K_i for the competitive inhibition. However, the tetrahedral intermediate [17] L-Ga3P-GAPN may not be able to follow the hydride ion transfer to the $NADP^+$ ring, probably because of its

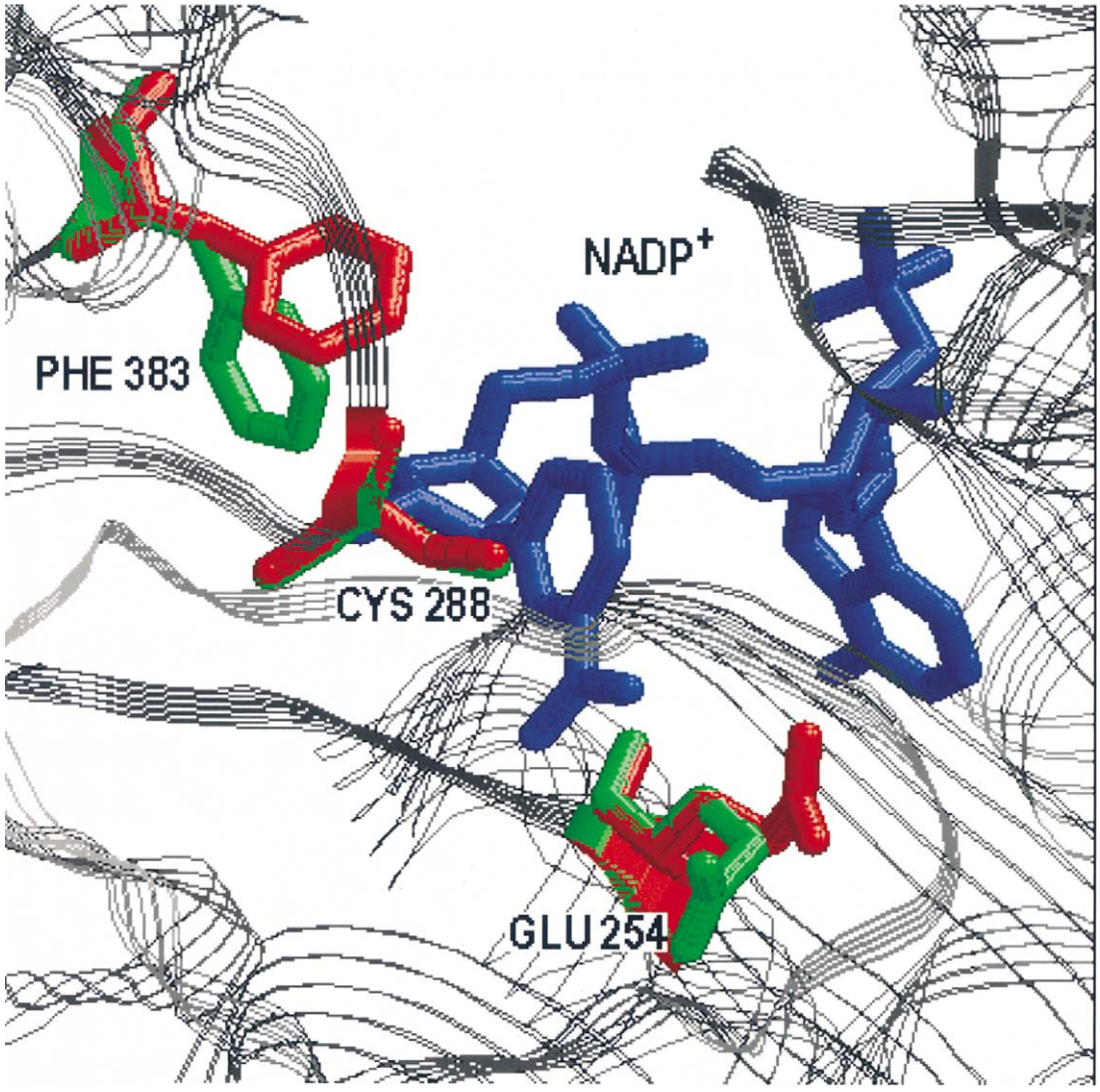


Fig. 3. Spatial relative distribution of $NADP^+$ (blue) and residues E254, C288, and F383 corresponding to GAPN from plants (green) or bacteria (red). Structural homology of spatial distribution of amino acid residues in the different GAPNs was established by modeling through the SWISS-MODEL [26–28] approach, taking as reference the crystallographic structure of the enzyme from *S. mutants* [19] and alignment of sequences of other bacteria and plant enzymes. Strands represent the protein backbone.

stereochemical configuration, and thus resulting in a dead-end derivative. Another possibility is that the hydride ion transfer still occurs but the thioester intermediate cannot be deacylated from the enzyme. From the latter options, L-Ga3P should behave kinetically as a non-competitive effector, as typical for enzyme inactivators when analyzed as reversible inhibitors [23,24]. Alternatively, a rapid release of the inactive enzyme-L-Ga3P adduct would allow the effect on V_{max} to be observed only at high concentrations of the substrate analogue.

Remarkably, GAPN from bacteria can use both enantiomeric forms of Ga3P as a substrate [18,25], showing a major difference between the plant and bacterial enzymes. From this, and to further investigate the causes for the distinctive behavior of the L-isomer, we made a comparison of the structural homology between GAPN from bacteria and plants. Modeling studies, by using the SWISS-MODEL [26–28] on the basis of the crystallographic structure of the enzyme from *S. mutants* and the known sequences of GAPN from several bacteria and plants, gave a tridimensional alignment of the different GAPN's residues, which was coincident in most of the different domains of the protein. Fig. 3 shows that residues E254 and F383 (numbers corresponding to the sequence of the celery leaf enzyme) exhibited a main difference in the spatial distribution in the region surrounding C288, when a comparison is made between GAPNs from bacteria respect to plants. The latter residue participates in catalysis (C284 in bacteria) by forming a thiohemiacetal intermediate with Ga3P [17].

Interestingly, amino acid E254 corresponds to the residue described as participating in the deacylation step of catalysis [18]. From the picture in Fig. 3, it is tempting to speculate that the tridimensional distribution of side chain residue F383 in plant GAPN (the aromatic ring in the plant enzyme is moved in an angle of about 120° respect of the residue in the bacterial enzyme) determines a steric constrict for the catalytic action of residue E254 or for the correct location of the nicotinamide ring of $NADP^+$ in a specific pocket in the protein. The steric restriction could be in a way that only the D-form of Ga3P can complete the catalytic sequence, whereas the L-isomer could still form the thiohemiacetal derivative with residue C288 but with a spatial distribution determining that the nicotinamide ring of $NADP^+$ and/or residue E254 is not close enough to the intermediate to allow the catalysis continue. The possibility that the different relative spatial distribution of these amino acid residues could affect the physicochemical properties of the catalytic site environment (i.e. changing pK_a values of key catalytic residues) should not be completely excluded.

A main conclusion of the present study is the high specificity of celery GAPN for effectors, as changes in the structure and steric configuration of the chemical moieties in the substrates highly affect the kinetic properties and the hysteretic behavior of the enzyme. The tridimensional model of the substrate binding region derived from the crystal structure of GAPN from *S. mutants* compared with that of the plant enzyme, suggests that the spatial distribution of key amino acid residues is critical to explain differences in substrate specificity observed between both enzymes. These residues are relevant targets for future studies to establish structure-to-function and regulation relationships in GAPN; to better understand similitudes and differences in kinetic and regulatory properties of the enzyme from different organisms.

Acknowledgements

This work was supported by grants from CONICET (PIP 0443/98) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT'99 No. 1-6074) to AAI. JIS is a fellow from Comisión de Investigaciones Científicas (CIC, Buenos Aires). DFGC and DMB are fellows from CONICET. AAI and FEP are staff scientists from the same institution.

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