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NAD^+ Mediated Differential Thermotolerance Between Chloroplastic and Cytosolic L-*myo*-Inositol-1-phosphate Synthase from *Diplopterygium glaucum* (Thunb.) Nakai

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Abstract: Relative thermotolerance of the enzyme, L-*myo*-inositol-1-phosphate synthase (MIPS; EC: 5.5.1.4), from the chloroplastic and cytosolic sources of *Diplopterygium glaucum* was studied. The purification involved streptomycin sulphate precipitation, ammonium sulphate fractionation, ion-exchange chromatography, and molecular sieve chromatography. After the final chromatography, 16.62% of chloroplastic and 13.47% of cytosolic MIPS could be recovered. Between 15°C and 55°C, the two forms of MIPS exhibited differential thermal stability, which is related to the presence of the MIPS co-factor, NAD^+. Added NAD^+ increased the lower thermotolerance of the chloroplastic MIPS and the removal of 'built-in' NAD^+ decreased the higher thermal stability of the cytosolic MIPS.

Keywords: *Diplopterygium glaucum*, L-*myo*-Inositol-1-phosphate synthase, *myo*-Inositol, Thermal stability, Built-in NAD

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INTRODUCTION

Inositol, a six-carbon cyclitol, is an essential component of eukaryotic and prokaryotic cells. *Myo*-inositol is the precursor of all inositol containing compounds, including phosphoinositides and inositol phosphates. The essential role of inositols in many cellular processes, including membrane formation, cell wall biogenesis, stress response, and signal transduction have been well documented.[1]

L-*myo*-Inositol-1-phosphate synthase (MIPS; EC: 5.5.1.4) catalyzes the conversion of glucose-6-phosphate (G-6-P) to L-*myo*-inositol-1-phosphate (I-1-P), the first and the rate limiting step in the production of *myo*-inositol. The cytosolic form of the MIPS has been reported from a number of sources, such as higher plants, animals, parasites, fungi, green algae, bacteria, and archea.[2–11]

In addition to the cytosolic forms of MIPS, the organellar form of the enzyme has been demonstrated in the chloroplasts of *Pisum sp., Vigna radiata, Euglena gracilis, Oryza sativa, and Phaseolus sp.*[12–15] A form of MIPS has also been found to be associated with the thayllakoidal membrane on *Phaseolus vulgaris*.[16] In the green plant chloroplasts, about 10% of the membrane phospholipids consist of phosphoinositides, which require an endogenous pool of *myo*-inositol for their biosynthesis. Impermeability of the chloroplast membrane to the cyclitol suggests that the plastid is the site for synthesis of myo-inositol.[13]

MIPS is known to function in varied biochemical activities, and also during stress, in both prokaryotic and eukaryotic organisms.[17–19] Of the 65 INO1 genes known so far that code for MIPS, the INO1 from *Archaeoglobus fulgidus*[20] has been shown to code for the only thermotolerant MIPS.[21] Earlier experiments have suggested that thermal stability of MIPS is associated with the NAD^+ bound to the enzyme.[22]

In the present communication, we describe two forms of MIPS, chloroplastic and cytosolic from the pteridophyte, *Diplopterygium glaucum* (Thunb.) Nakai. The two forms of this enzyme from the plant have been partially purified and compared with respect to their biochemical properties. A considerable difference in thermal stability in MIPS from these cellular compartments was noted. The property of the enzyme and its relationship with bound NAD^+ has been dealt with herein.

EXPERIMENTAL

Chemicals

G-6-P, β-NAD, imidazole, and *myo*-inositol were purchased from Sigma. 2-Mercaptoethanol, ammonium sulphate, Tris, and EDTA were from E. Merck, India Ltd.; DEAE-cellulose (DE-52) from SRL, India; Hexylagarose from Miles-Yeda; and BioGel A-0.5 m was obtained from Bio-Rad. All other chemicals used were of analytical reagent grade.

Plant Material

Fresh specimens of pteridophyte, *Diplopterygium glaucum* (Thunb.) Nakai (*D. glaucum*) were collected from their natural habitat in the Darjeeling hills (2134 m asl.) in the Eastern Himalayas of India. Reproductive pinnules from *D. glaucum* fronds were separated; they were washed three times in order to purify and characterize cytosolic MIPS. The sample was homogenized in a chilled mortar and pestle in two volumes of 50 mM Tris-acetate buffer (pH 7.5) containing 0.2 mM ME. The crude homogenate was centrifuged at 1,000 *g* for 5 minutes in a Plasto Crafts Superspin-R centrifuge. The supernatant was again centrifuged at 11,400 *g* for 20 minutes and the clear supernatant was collected.

Chloroplasts of *D. glaucum* were isolated following the method of Hatchtel[23] with minor modifications. Juvenile vegetative pinnules of *D. glaucum* were collected fresh and washed several times with distilled water, and then placed in plastic bag in a refrigerator for a few hours until they become turgid. The material was then homogenized with 2 volumes of 20 mM Tris-acetate (pH 7.0) containing 0.35 M sucrose, 10 mM $MgCl_2$, 10 mM KCl, 1 mM ME, 10 mM sodium ascorbate, and 2 mL (per 100 mL of buffer) of chicken-egg albumin. The supernatant obtained after centrifugation at 435 *g* for 5 minutes was spun at 2850 *g* for 15 minutes. The resultant chloroplast pellet was washed 3 times with the homogenizing medium. The purity of the isolated chloroplasts was checked by the ability of the preparation to carry out a characteristic Hill reaction using DCPIP as the electron acceptor, along with the determination of total chlorophyll content.[24] The isolated chloroplasts were washed with chilled 50 mM Tris-acetate buffer (pH 7.0) containing 0.2 mM ME. The buffer-washed chloroplasts were homogenized in a chilled mortar and pestle with 3 volumes of 50 mM Tris-acetate (pH 7.0) containing 0.2 mM ME and centrifuged at 11,400 *g* for 20 minutes. The clear supernatant was recovered from the centrifuge tubes.

Partial Purification of MIPS

To purify MIPS, the clear supernatant (11,400 *g* supernatant) fraction obtained from the cytosol and chloroplast preparations were subjected to 2% and 1% (w/v) streptomycin sulphate treatment, respectively, with constant stirring. The mixtures were kept in ice bucket at 0°C for 15 minutes and then centrifuged at 11,400 *g* for 15 minutes. The streptomycin sulphate treated supernatant fractions were collected and fractionated with 0–70% (0–80% in case of chloroplastic preparation) ammonium sulphate. The precipitated protein was dissolved in a minimal volume of the extraction buffer and dialyzed against the same buffer. The ammonium sulphate fractions were recovered from the dialysis bags and adsorbed onto DEAE-cellulose for

2 hours; the preparations were loaded into glass columns. The adsorbed proteins were eluted from the column with a linear gradient of 0 to 0.5 M KCl. The active DEAE-cellulose fractions were chromatographed on a hexylagarose column and eluted with 50 mM imidazole-HCl (pH 7.5) containing 0.2 mM ME. The active hexylagarose fractions were loaded into a column of BioGel A-0.5 m and the proteins were eluted with the extraction buffer. The pooled active fractions (BioGel A-0.5 m fraction) were concentrated and used as the ultimate enzyme source for further analysis.

Assay of MIPS Activity

The MIPS activity from both the chloroplastic and the cytosolic sources of *D. glaucum* was assayed by the procedure of Barnett et al.[25] with slight modifications.[13] The assay mixture contained 50 mM Tris-acetate (pH 7.5), 14 mM NH_4Cl, 0.8 mM NAD^+, 5 mM ME, 5 mM G-6-P, and an appropriate aliquot (100-200 μg) of enzyme protein in a total volume of 500 μL. After incubation at 37°C for 1 h, the reaction was terminated by addition of 200 μL of 20% (v/v) chilled TCA. An equal volume of 0.2 M $NaIO_4$ was added to the deproteinized supernatant, followed by a second incubation at 37°C for 1 h for the oxidation of MIPS reaction product, I-1-P causing the release of inorganic phosphate. The excess periodate was destroyed with 1M Na_2SO_3. Simultaneously, non-periodate controls, in which $NaIO_4$ and Na_2SO_3 treatments were omitted, were also run. The activity of the enzyme was determined by estimating the product-specific release of inorganic phosphate from I-1-P by the MIPS reaction.

Inorganic phosphate was determined by the method of Chen et al.[26] with slight modifications. A freshly prepared Pi-reagent (2.8 mL.) containing H_2SO_4 (6 N), ascorbic acid (10%, w/v), chilled ammonium molybdate (2.5%, w/v), and H_2O mixed in a 1 : 1 : 1 : 2 ratio was added to the reaction mixture and incubated at 37°C for 1 h. The blue color which developed was measured at 820 nm in a Beckman DU-64 spectrophotometer. The released inorganic phosphate was quantified with a standard curve prepared using K_2HPO_4. Protein was determined according to the method of Bradford.[27] The protein content in fractions obtained from column chromatography was also determined by measuring absorbance at 280 nm.

Removal of Endogenous NAD

Endogenous NAD was removed from MIPS according to the method of Barnett et al.[28] Partially purified MIPS was stirred with phosphate-free activated charcoal (100 mg/mL) for 15 minutes at 0°C and centrifuged at 5000 *g* for 10 minutes. The resultant supernatant was used as the endogenous NAD^+ free enzyme.

RESULTS

Occurrence of Two Forms of MIPS in *D. glaucum*

While studying the MIPS activity from the vegetative and the reproductive pinnules of *D. glaucum*, it was found that the enzyme is functional both in the homogenate as well as the clear supernatant obtained after centrifugation of the homogenized material at 11,400 *g*. The homogenate fraction exhibited a much higher activity of the enzyme, as compared to the clear supernatant. Furthermore, the degree of enzyme activity was much higher (1.5-fold, approx.) where the vegetative pinnules (chloroplast dominating) are the enzyme source (Table 1). This indicates the occurrence of the chloroplastidial form of the enzyme, in addition to its cytosolic form. The authenticities of the synthase from both the particulate and the cytosolic sources were established by the fact that MIPS from both sources were absolutely dependent on G-6-P and NAD^+ as the substrate and the co-enzyme, respectively. Identification of the reaction product as I-1-P was done by enzymatic assay of the putative synthase product by its specific cleavage by inositol-monophosphatase.[13]

Partial Purification of Chloroplastic and Cytosolic MIPS

The particulate enzyme (chloroplastic) and the cytosolic enzyme were isolated and partially purified from the vegetative and the reproductive pinnules of *D. glaucum*, respectively. The summary of partial purification of the enzyme is given in Table 2. It is evident, from the results that, from *D. glaucum*, the chloroplastic MIPS could be purified to about 28-fold over the homogenate fraction with 16.62% recovery. However, the cytosolic MIPS could be purified to about 81-fold with 13.47% recovery.

Table 1. L-*myo*-Inositol 1-phosphate synthase activity in vegetative pinnules of *Diplopterygium glaucum* as compared to reproductive pinnules of the same species. Specific activity defined as [μ mol I-1-P produced $(mg)^{-1}$ $protein^{h-1}$]

Plant tissue type	Enzyme source	Total protein extracted (mg g^{-1} FW)	Specific activity [μ mol I-1-P produced $(mg)^{-1}$ $protein^{h-1}$]
Vegetative pinnules	Homogenate	1.329 ± 0.168	0.076 ± 0.005
	11,400 g supernatant	0.886 ± 0.049	0.056 ± 0.007
Reproductive pinnules	Homogenate	1.684 ± 0.110	0.118 ± 0.014
	11,400 g supernatant	1.138 ± 0.075	0.142 ± 0.005

FW = Fresh weight, Means ± SE.

ry of partial purification of chloroplastic and cytosolic L-*myo*-inositol 1-phosphate synthase from *Diplopterygium glaucum.*
nd total enzyme activity defined as [μ mol I-1-P produced $(mg)^{-1}$ $protein^{h-1}$]

Specific activity		Total activity		Recovery (%)		Purification-fold	
Chl.	Cyt.	Chl.	Cyt.	Chl.	Cyt.	Chl.	Cyt.
0.134 ± 0.031	0.120 ± 0.020	1.91 ± 0.13	13.43 ± 1.24	100 ± 7.94	100 ± 15.29	1.00 ± 0.12	1.00 ± 0.15
0.148 ± 0.016	0.130 ± 0.008	1.54 ± 0.15	12.24 ± 1.68	80.40 ± 10.83	91.13 ± 11.34	1.10 ± 0.14	1.09 ± 0.30
0.163 ± 0.008	0.140 ± 0.026	1.50 ± 0.10	11.51 ± 1.27	78.69 ± 5.80	85.70 ± 7.68	1.21 ± 0.18	1.18 ± 0.28
0.168 ± 0.015	0.160 ± 0.042	1.39 ± 0.11	10.74 ± 1.76	72.94 ± 6.39	79.76 ± 9.19	1.25 ± 0.12	1.29 ± 0.17
0.464 ± 0.026	1.213 ±0.150	0.90 ± 0.15	7.07 ± 1.30	46.96 ± 4.76	52.66 ± 6.19	3.45 ± 0.53	10.01 ± 1.61
1.119 ± 0.116	5.020 ± 0.539	0.76 ± 0.08	5.28 ± 0.50	39.77 ± 1.79	39.34 ± 2.44	8.32 ± 1.31	41.40 ± 1.93
3.794 ± 0.155	9.981 ± 2.155	0.31 ± 0.05	1.81 ± 0.01	16.62 ± 3.30	13.47 ± 1.08	28.21 ± 2.08	80.99 ± 4.19

astic, Cyt. = cytosolic.

Effect of Incubation Temperature on MIPS Activity

The chloroplastic and cytosolic *D. glaucum* MIPS from the BioGel A 0.5 m fraction were incubated separately for 1 h at temperature ranges of 15°C to 55°C in presence of standard assay mixture. Activities of the MIPS from both the sources were negligible at the two extremes of 15°C and 55°C. However, in both the cases, maximum activity was recorded at 35°C, followed by a sharp decline (Fig. 1).

Effect of Preincubation at Different Temperatures

The partially purified MIPS from chloroplastic and cytosolic sources of *D. glaucum* were preincubated for 5 minutes in a water bath at different temperatures between 15°C and 55°C prior to the actual enzyme incubation at 37°C for 1 h to determine the MIPS activity in the presence of the usual components of the assay mixture. The specific activity, thus obtained for each set, was plotted against preincubation temperature. The results revealed the preincubation temperature maxima for the chloroplastic and cytosolic enzyme forms of *D. glaucum* to be 25°C and 35°C, respectively. The difference in thermal stability between the enzymes from the two sources thus became evident (Fig. 2).

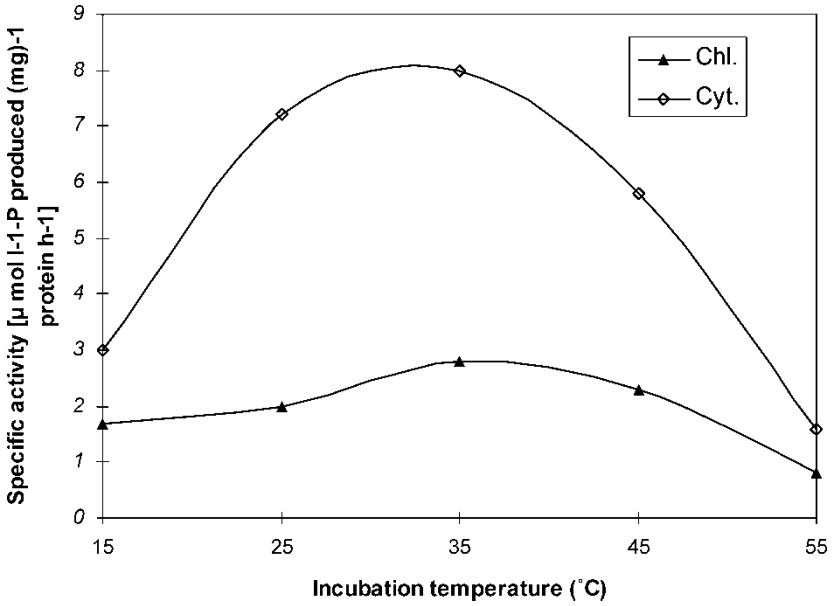

Figure 1. Chloroplastic (Chl.) and cytosolic (Cyt.) L-*myo*-inositol 1-phosphate synthase activity in *Diplopterygium glaucum* at different incubation temperatures.

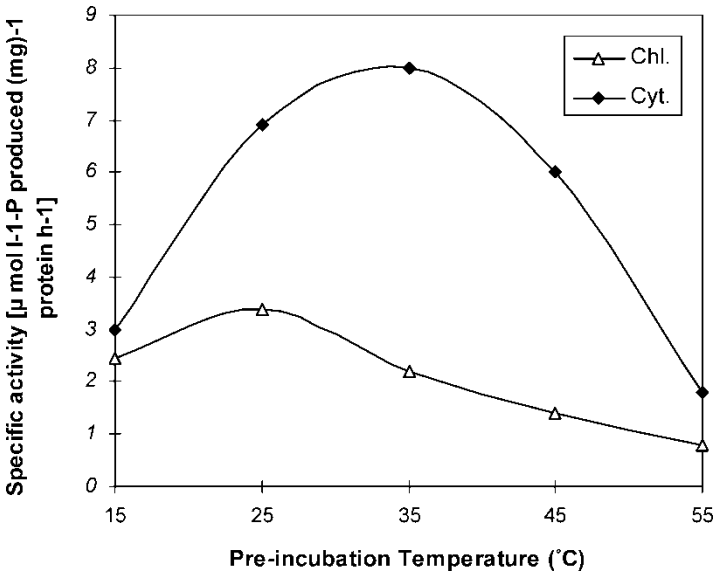

Figure 2. Effect of different preincubation temperature on chloroplastic (Chl.) and cytosolic (Cyt.) L-*myo*-inositol 1-phosphate synthase activity in *Diplopterygium glaucum* in the presence of all of the components of the assay mixture.

Effect of Assay Components on Thermal Sensitivity of *D. glaucum* MIPS

In presence of all the components of the assay mixture, the MIPS from both the sources show identical temperature maxima profiles, i.e., both the enzymes showed maximum activity at 35°C. This proved that a component in the assay system, either alone or together with others, confers thermal stability to the chloroplastic enzyme, thereby making it quantitatively similar to the cytosolic enzyme.

To determine the nature of the 'responsible component(s)' for conferring thermal stability to the chloroplastic MIPS, preincubation of the enzyme at different temperatures was performed along with the 'drop-out assay mixture' on which one of the components of the total assay mixture, namely, Tris-acetate buffer, NH_4Cl, NAD^+, ME, or G-6-P, was omitted in a series of experiments. Results from such experiments revealed that the lack of NAD^+ in the assay mixture caused the lowering of temperature maxima of the chloroplastic MIPS from 35°C. The other components of the assay mixture, either alone or collectively, did not show such responses. The pattern of the cytosolic MIPS activity remained unaltered, irrespective of the presence or absence of these factors. Since MIPS from different sources have been reported to contain 'built-in' NAD^+, the presence of enzyme bound NAD^+ is the most probable reason for differential thermotolerance between the two enzymes.[28]

Removal of 'Built-in' NAD^+ and Its Effect on MIPS Activity

When the enzyme from both the sources were treated with activated charcoal, a process known to remove endogenous NAD^+, the preincubation temperature maxima for the cytosolic MIPS dropped to 25°C from the usual 35°C. However, with the same procedure, the chloroplastic enzyme remained unaltered at 25°C. In contrast to the untreated chloroplastic enzyme, both the cytosolic and chloroplastic charcoal treated enzymes showed variable responses in gaining sharp temperature maxima at 35°C when NAD^+ (0.8 mM) was added to the system (Fig. 3). Nevertheless, increased activities of both the enzymes were evident up to 45°C.

The fact that the *D. glaucum* MIPS from the two sources differed considerably in their endogenous NAD^+ content is evident from the experiment presented in Table 3. The charcoal-untreated cytosolic enzyme retains about 72% of its activity in absence of added NAD^+, whereas only about 54% of the activity was retained by the chloroplastic MIPS under similar conditions. In contrast, upon charcoal treatment, both the enzymes worked identically and each of them lost about 49–59% of its activity in the absence of NAD^+, while 66–74% of the activity was regained when NAD^+ (0.8 mM) was added (Table 3).

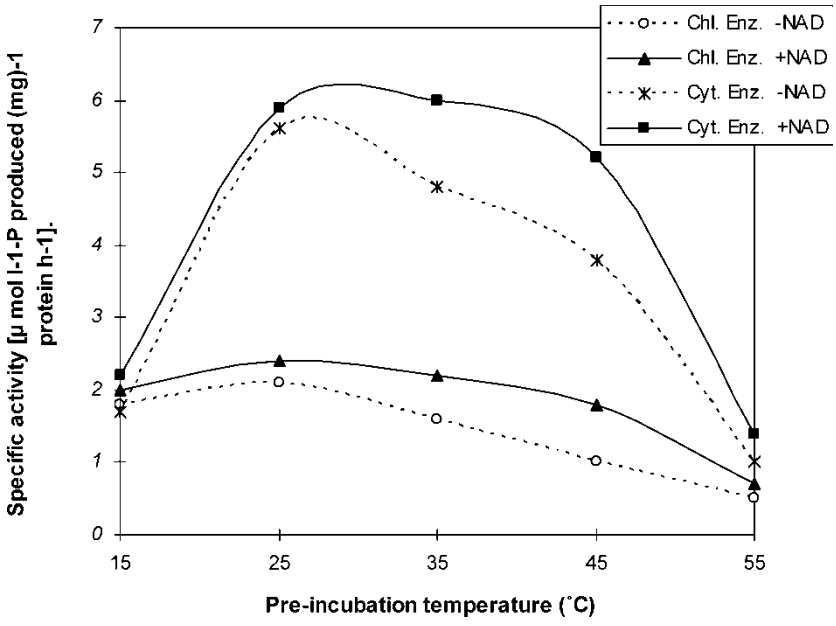

Figure 3. Effect of different preincubation temperatures on chloroplastic (Chl.) and cytosolic (Cyt.) L-*myo*-Inositol 1-phosphate synthase activity in *Diplopterygium glaucum* in the absence of NAD^+ or in the presence of added NAD^+.

Table 3. Alteration of *Diplopterygium glaucum* MIPS activity on the removal of 'built-in' NAD^+ by activated charcoal and its recovery

Enzyme source	Conditions	Specific activity [μ mol I-1-P produced $(mg)^{-1}$ $protein^{h-1}$]	Enzyme activity[a] (%)	Drop in activity (%)
Chloroplastic	Untreated			
	$-NAD^+$	1.508 ± 0.117	53.56 ± 7.75	46.44 ± 6.71
	$+NAD^+$	2.816 ± 0.203	100.00 ± 7.21	0.00
	Charcoal treated			
	$-NAD^+$	1.143 ± 0.061	40.59 ± 5.37	59.41 ± 7.85
	$+NAD^+$	1.866 ± 0.130	66.25 ± 6.99	33.75 ± 3.56
Cytosolic	Untreated			
	$-NAD^+$	5.873 ± 0.319	72.44 ± 5.43	27.56 ± 2.06
	$+NAD^+$	8.107 ± 1.028	100.00 ± 12.68	0.00
	Charcoal treated			
	$-NAD^+$	4.102 ± 0.126	50.60 ± 3.07	49.40 ± 2.99
	$+NAD^+$	6.022 ± 0.540	74.27 ± 8.97	25.73 ± 3.10

[a]100% activity corresponds to the activity of the untreated enzymes(s) in presence of optimal concentration of NAD^+ (0.8 mM).

DISCUSSION

Studies with MIPS of pteridophytic origin have been started by Chhetri et al.[29,30] During the search for MIPS activity, separate from the vegetative, as well as reproductive pinnules of *D. glaucum*, it was found that the enzyme activity was higher in the homogenate fraction over the low speed supernatant fraction, especially in case of vegetative pinnules (Table 1). This points towards the possibility of the existence of polymorphic forms of the enzyme in *D. glaucum*, where at least one form may be membrane bound. It has been known, from previous investigations, that one form of this enzyme is located in chloroplasts.[12,13]

During the present investigation, appreciable MIPS activity was determined in the chloroplasts which were isolated from the vegetative pinnules of *D. glaucum*. Thus, the occurrence of chloroplastic MIPS in addition to the cytosolic MIPS in *D. glaucum* became evident. Ultimately, the particulate form of MIPS present in chloroplast of *D. glaucum* has been purified along with the cytosolic form (Table 2).

After having a partially purified enzyme preparation, it was determined that the cytosolic and chloroplastic forms of the enzyme showed differences in thermal sensitivity. Such differential thermotolerance was co-related to the presence of 'built-in' NAD^+ in *D. glaucum*. A distinct difference in

the preincubation temperature maxima for the two enzyme forms was noted. Further, it was revealed that the MIPS co-factor NAD^+ conferred thermal stability to the chloroplastic *D. glaucum* enzyme. Obviously, in the case of cytosolic *D. glaucum* MIPS, thermal stability was due to the NAD^+ bound to the enzyme. Thus, when the endogenous NAD^+ was removed from the cytosolic MIPS by activated charcoal treatment, preincubation temperature maxima dropped to 25°C, the characteristic of the chloroplastic enzyme from *D. glaucum*. From the present studies, it was also proved that the cytosolic and chloroplastic *D. glaucum* MIPS differ substantially with respect to enzyme bound NAD^+ content, which is responsible for thermal stability of the enzyme. Considering the molecular architecture of MIPS, in the case of the cytosolic enzyme, we cannot rule out the possibility that at least some of the region of the enzyme molecule may be stabilized by full occupancy of NAD^+.[31] The chloroplastic enzyme was more dependent upon added NAD^+ for optimum activity, thus suggesting a higher content of endogenous NAD^+ in cytosolic MIPS.

The causes of enhanced thermostability of the archeal MIPS may be due to the presence of NAD^+ and inorganic phosphate in the active sites of its subunits. Moreover, in *Archaeoglobus fulgidus*, the enhanced thermostability of MIPS, as compared to that from yeast, is consistent with the deletion of a number of surface loops that result in a significantly smaller protein.[32] The cytosolic and chloroplastic MIPS are similar in their biochemical, immunological and protein sub-unit characteristics and differ only in the organization of the subunits in the native holoenzymes. Typically, the native molecular weight of the cytosolic MIPS was found to be 179 to 200 kDa and that for native chloroplast enzyme varied between 248 and 266 kDa.[14] Thermal sensitivity of MIPS from animal sources is either fully or partially dependent on the endogenous NAD^+ content of the enzyme.[22] However, in the plant system, no such phenomenon was established, so far.

The present study suggests that the differences in thermal stability between the chloroplastic and cytosolic MIPS from *D. glaucum* is associated with the enzyme bound NAD^+; whether the influence of other proteins, if any, present in the partially purified enzyme preparation, may influence the thermal sensitivity of the enzyme or not is still an open question. Of course, the predominant role of NAD^+ in conferring thermal stability is conclusive.

ABBREVIATIONS

DCPIP = Dichlorophenol indophenol; G-6-P = D-glucose-6-phosphate; I-1-P = Inositol-1-phosphate; ME = 2-mercaptoethanol; MIPS = L-*myo*-inositol-1-phosphate synthase.

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