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MYO-INOSITOL DEHYDROGENASE FROM THE ACIDO- AND THERMOPHILIC RED ALGA *GALDIERIA SULPHURARIA*

ROBERT STEIN, CLAUS SCHNARRENBERGER and WOLFGANG GROSS

Institut für Pflanzenphysiologie und Mikrobiologie, Freie Universität Berlin, Königin-Luise-Str. 12-16, D-14195 Berlin, Germany

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Key Word Index—*Galdieria sulphuraria*; Rhodophyta; *myo*-inositol dehydrogenase; *scyllo*-inosose; purification; properties.

Abstract—A NAD-dependent *myo*-inositol dehydrogenase (EC 1.1.1.18) has been purified from the acido- and thermophilic red alga *Galdieria sulphuraria*. This enzyme catalyses the reversible oxidation of *myo*-inositol to *scyllo*-inosose (2-keto-inositol). The activity with *scyllo*-inosose and NADH was *ca* 75-times higher than with *myo*-inositol and NAD. At pH 8.0 the equilibrium of the reaction strongly favours the production of *myo*-inositol. The K_m values for *myo*-inositol and *scyllo*-inosose were 430 mM and 1.3 mM, respectively. The dehydrogenase is specific for *myo*-inositol and *scyllo*-inosose. The enzyme was purified about 205-fold to apparent homogeneity with a specific activity of 63 μkat mg protein^{-1} with *scyllo*-inosose as substrate. The M_r of the subunits was 42 000 and of the native enzyme *ca* 180 000.

INTRODUCTION

A large number of different cyclitols occur in plants [1]. The most widespread of these compounds is *myo*-inositol, being a constituent of the phosphoinositides of membranes, the phosphate storing phytic acid, the galactosyl donor galactinol, and of IAA derivatives. Since *myo*-inositol is also the precursor for compatible solutes (pinitol, ononitol) and pectins during seed germination, it is obvious that the metabolism of this compound is very complex. The synthesis of *myo*-inositol proceeds from glc-6-P via *myo*-inositol-1-P, while the degradation involves a *myo*-inositol oxygenase reaction, yielding glucuronic acid [2]. An alternative pathway has been proposed for the higher plant *Calycanthus occidentalis* [3] where *scyllo*-inosose is the substrate for *myo*-inositol synthesis (Fig. 1). In the red alga *Galdieria sulphuraria* we have found a highly active *myo*-inositol dehydrogenase which interconverts *scyllo*-inosose and *myo*-inositol. Here we report the purification and some properties of this NAD-dependent dehydrogenase.

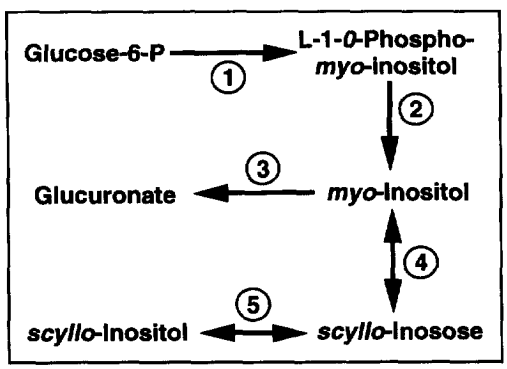

Fig. 1. Pathways of *myo*-inositol metabolism in plants. (1) *myo*-Inositol-1-phosphate synthase; (2) *myo*-inositol-1-phosphate phosphatase; (3) *myo*-inositol oxygenase; (4) NAD-dependent *myo*-inositol dehydrogenase; (5) NADP-dependent *scyllo*-inositol dehydrogenase.

RESULTS

The red alga *Galdieria sulphuraria* exhibits high activity of a NAD-dependent *myo*-inositol dehydrogenase (EC 1.1.1.18). This enzyme is apparently specific for *myo*-inositol and NAD. *scyllo*- and *epi*-Inositol gave *ca* 5% of the rate obtained with *myo*-inositol. Other polyols (D-mannitol, D-sorbitol, dulcitol, L-fucitol, xylitol, adonitol, D-arabitol, L-arabitol, D-, L-threitol, *meso*-erythritol) as well as sugars (D-glucose, D-xylose) did not serve as substrate. The K_m for *myo*-inositol was 430 mM and the enzyme showed increasing activity from pH 6 to 10. The product of the reaction with *myo*-inositol as substrate was *scyllo*-inosose (Fig. 2). In the reverse reaction the activity of the enzyme with *scyllo*-inosose and NADH was *ca* 75-times higher as with *myo*-inositol and NAD. The equilibrium at pH 8.0 clearly favoured the production

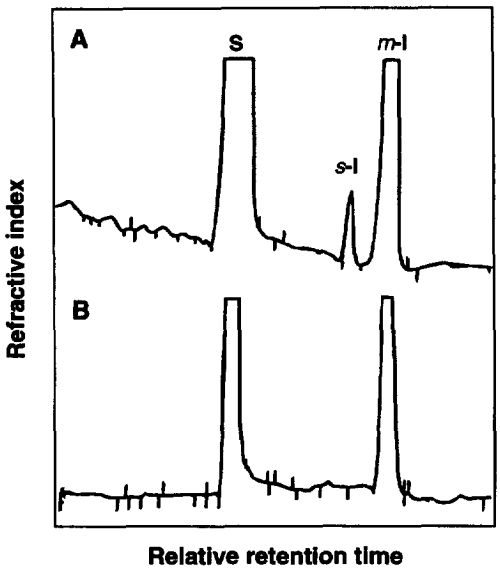

Fig. 2. Separation and identification of the reaction product of the *myo*-inositol dehydrogenase with *myo*-inositol as substrate on a BC-100 Ca^{2+} column. (A) Elution profile of a complete reaction mixture after 30 min incubation. (B) Elution profile of a reaction mixture without NAD after 30 min incubation. S—solvent; *s*-I—*scyllo*-inosose; *m*-I—*myo*-inositol.

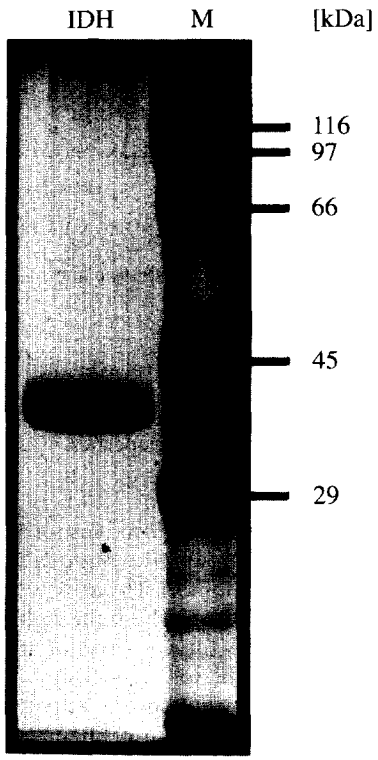

Fig. 3. Protein pattern after SDS-PAGE and silver staining of the *myo*-inositol dehydrogenase purified by size-exclusion chromatography. M—marker proteins; IDH—*myo*-inositol dehydrogenase.

of *myo*-inositol. The conversion rate for *epi*-inosose was only 5–10% of that with *scyllo*-inosose. The K_m for *scyllo*-inosose was 1.3 mM and the pH optimum was 8.0 with half maximal activity at pH 5.5 and 9.5.

The *myo*-inositol dehydrogenase was purified 205-fold to apparent homogeneity by conventional column chromatography. SDS-PAGE of the purified enzyme showed a single band of 42 kDa (Fig. 3), while the M_r of the native enzyme was 180 000 and 160 000 as estimated by size-exclusion chromatography and by density gradient centrifugation, respectively. This suggests that the dehydrogenase is a homotetramer. When crude extracts were subjected to non-denaturing PAGE and subsequently stained for *myo*-inositol dehydrogenase activity, a minor and a major band were detectable (data not shown). The major band belonged to the *myo*-inositol dehydrogenase while the minor band was due to a xylitol dehydrogenase with a broad substrate spectrum [4]. The *myo*-inositol dehydrogenase was stable during purification, even 2 hr incubation at 50° did not lead to inactivation.

We did not observe significant changes in the specific activity of the *myo*-inositol dehydrogenase when cells were grown auto- or heterotrophically on glucose, xylitol and mannitol, or when cells were stressed either by changing the growth temperature from 25 to 50° or increasing the salinity of the medium to 5% NaCl. Also, the activity of the dehydrogenase was unaffected by phosphate starvation and subsequent resupply (data not shown).

DISCUSSION

The red alga *Galdieria sulphuraria* is able to grow heterotrophically on at least 27 different sugars and polyols. *Myo*-inositol, however, does not support growth of the alga [5]. Investigating the polyol

Table 1. Purification of the *myo*-inositol dehydrogenase

	Total protein (mg)	Activity* (μkat)	Sp. act.* (μkat mg^{-1})	Yield (%)	Purification (-fold)
Crude extract	273	1.11	0.003	100	1
DEAE-Fractogel	45.9	0.43	0.010	39	2
Matrex gel blue A	18.0	0.65	0.037	58	9
Hydroxyapatite	2.2	0.27	0.188	25	46
Superdex 200	0.2	0.14	0.837	12	205

*Measured with *myo*-inositol.

dehydrogenases in *Galdieria sulphuraria*, we found an NAD-dependent oxidoreductase apparently specific for *myo*-inositol yielding *scyllo*-inosose as product. We purified this enzyme to apparent homogeneity. Although we tested a variety of polyols and sugars as substrates, only *scyllo*-inosose and *myo*-inositol served as substrates. A *myo*-inositol dehydrogenase has been found in mammals [6], insects [7], yeasts [8] and bacteria [9, 10]. The enzyme has been purified and extensively characterised from *Bacillus subtilis* [10]. Similar to the enzyme from *G. sulphuraria*, the bacterial dehydrogenase is a tetramer with a subunit size of 36.5 kDa. The K_m values for *myo*-inositol and *scyllo*-inosose as well as the pH optima are also very similar for the algal and bacterial enzyme. In contrast to the enzyme from *G. sulphuraria*, the dehydrogenase from *Bacillus* also reacts with D-glucose (25%) and D-xylose (14%).

In yeast and bacteria the enzyme functions in the utilisation of exogenous *myo*-inositol. The role of the enzyme in animals is not clear [6], although *myo*-inositol and *scyllo*-inosose are normal constituents of mammalian tissues [11]. It has been proposed that an NAD-dependent *myo*-inositol dehydrogenase and an NADP-dependent *scyllo*-inositol dehydrogenase facilitate the interconversion of these two cyclitols via *scyllo*-inosose as an intermediate [6, 7] (Fig. 1). The same pathway has also been proposed for the higher plant *Calycanthus occidentalis* [3] on the grounds of feeding experiments with various cyclitols, however, the respective enzymes have not been studied in this plant. In contrast, the biosynthesis of *myo*-inositol in plants is well documented. In this pathway, *myo*-inositol is synthesized from glucose-6-P via *myo*-inositol-1-P and converted to glucuronate by an oxygenase [2].

The *myo*-inositol dehydrogenase is apparently not part of the reaction sequence from *myo*-inositol to *scyllo*-inositol in *Galdieria sulphuraria* because we did not detect activity of an NADP-dependent *scyllo*-inositol dehydrogenase in crude extracts, nor did the *myo*-inositol dehydrogenase use NADP as cosubstrate (data not shown). In addition, neither *myo*-inositol nor *scyllo*-inosose were detectable in extracts of the alga by HPLC (data not shown). Therefore, the function of the *myo*-inositol dehydrogenase remains unclear. However, the high specific activity of the *myo*-inositol dehydrogenase in crude extracts of the alga suggests an important metabolic function.

EXPERIMENTAL

Galdieria sulphuraria (Galdieri) Merola (strain 074G) was grown as described previously [5].

Purification of the myo-*inositol dehydrogenase*. The prepn of crude extracts is described in ref. [12]. The membrane-free extract was loaded onto a Fractogel TSK-DEAE-650(s) column (20 × 2.5 cm) equilibrated with 20 mM Tris–HCl, pH 8.5. The column was washed with 150 ml buffer. The *myo*-inositol dehydrogenase activity was recovered in this wash fraction. The sample was dialysed against MOPS–KOH, 20 mM, pH 7.5 containing 0.5 mM 2-mercaptoethanol overnight and loaded onto a dye-ligand column (Matrex gel blue A; Amicon corp., MA, USA)(6 × 2.5 cm) equilibrated with the same buffer. The column was washed with 100 ml buffer. Proteins were eluted by a linear gradient from 0 to 1 M KCl. Fractions containing dehydrogenase activity were pooled, dialysed against 10 mM K-Pi, pH 7.0 containing 0.5 mM 2-mercaptoethanol overnight and loaded onto a hydxoxyapatite column (Econo-Pak HTP; 5 ml; Bio-Rad) equilibrated with the same buffer. After a washing step (15 ml) the proteins were eluted by a K-Pi gradient of 0.01–0.3 M. The peak fractions were pooled and aliquots of 2 ml were applied onto a size-exclusion column (Superdex 200 HiLoad, 16 × 600 mm; Pharmacia) equilibrated with 20 mM MOPS–KOH, pH 7.5, and 0.25 M NaCl.

Assay of myo-*inositol dehydrogenase*. The reaction mixt. contained 50 mM Tris–HCl, pH 9.5, 0.75 mM NAD, 25 mM *myo*-inositol, and enzyme. In the reverse reaction the assay contained 20 mM K-Pi buffer, pH 8.0, 0.3 mM NADH, 2 mM *scyllo*-inosose, and 50 μl of enzyme. The reaction was started by the addition of substrate. For estimating the equilibrium of the reaction, the enzyme was incubated overnight with 1 mM *scyllo*-inosose and 1 mM NADH as well as with *myo*-inositol and NAD at the same concn at pH 8.0. The reaction products were analysed by HPLC as described (see below). Other enzymes were assayed according to published methods: catalase, hexokinase, malate dehydrogenase, peroxidase, glucosephosphate isomerase [13].

Identification of reaction products. The reaction mixture was the same as for the photometric test. After 3 hr at room temp. the reaction was terminated by the addition of 3 vol. of EtOH (96%). Controls were done without NAD or without substrate. Samples were frozen for 20 min at −20°, thawed, and centrifuged for 10 min at 13 000 *g*. The supernatant was collected and evapd under red. pres. (SpeedVac, Univapo 150-H). The residue was dissolved in 2 × -dist. H_2O, filtered through nylon filter (0.2 μm), and aliquots of 20 μl were analysed on an HPLC-column (BC-100, Ca^{2+}, 7.8 × 300 mm; Benson Co., Reno, U.S.A.). The mobile phase was 2 × distilled H_2O and the column temp. was 85°. The refractive index of the effluent was monitored at 50°. Compounds were identified by comparison with the retention time of standard sugars and polyols.

Determination of M_r. The M_r of the subunit of the dehydrogenase was estimated from SDS-PAGE using 10%-slab gels [14]. Proteins on gels were stained with *silver* according to ref. [15]. The M_r of the native *myo*-inositol dehydrogenase was estimated by the sedimentation velocity in density gradients [12] and by size-exclusion chromatography on a Superdex 200 HiLoad column (16 × 600 mm; Pharmacia) [16].

Protein was determined by the Coomassie Bt Blue

method [17] or by the method of ref. [18]. K_m was determined from Lineweaver–Burk plots.

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REFERENCES

1. Drew, E. D., in *Storage Carbohydrates in Vascular Plants*, ed. D. H. Lewis. Cambridge University Press, Cambridge, 1984, p. 133.
2. Loewus, F. A. and Loewus, M. W., *Annual Review of Plant Physiology*, 1983, **34**, 137.
3. Scholda, R., Billek, G. and Hoffmann-Ostendorf, O., *Biological Chemistry Hoppe-Seyler*, 1964, **339**, 28.
4. Stein, R., Gross, W. and Schnarrenberger, C., *Planta* (in press).
5. Gross, W. and Schnarrenberger, C., *Plant Cell Physiology*, 1995, **36**, 633.
6. Hipps, P. P., Eveland, M. R., Laird, M. H. and Sherman, W. R., *Biochimica et Biophysica Research Communications*, 1976, **68**, 1133.
7. Candy, D. J., *Biochemistry Journal*, 1967, **103**, 666.
8. Vidal-Leiria, M. and Van Uden, N., *Biochimica Biophysica Acta*, 1973, **293**, 295.
9. Berman, T. and Magasanik, B., *Journal of Biological Chemistry*, 1966, **241**, 800.
10. Ramaley, R., Fujita, Y. and Freese, E., *Journal of Biological Chemistry*, 1979, **254**, 7684.
11. Sherman, W. R., Stewart, M. A., Simpson, P. C. and Goodwin, S. L., *Biochemistry*, 1968, **7**, 819.
12. Gross, W. and Schnarrenberger, C., *European Journal of Biochemistry*, 1995, **234**, 258.
13. Bergmeyer, H. U., *Methoden der enzymatischen Analyse*. Verlag Chemie, Weinheim, 1970.
14. Laemmli, U. K., *Nature*, 1970, **227**, 680.
15. Blum, H., Beier, H. and Gross, H. J., *Electrophoresis*, 1987, **8**, 93.
16. Prosselkov, P. V., Gross, W., Igamberdiev, A. U. and Schnarrenberger, C., *Physiologica Plantarum*, 1996, **98**, 753.
17. Bradford, M. M., *Analytical Biochemistry*, 1976, **72**, 248.
18. Warburg, O. and Christian, W., *Biochemische Zeitschrift*, 1941, **310**, 384.