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STUDIES ON *myo*-INOSITOL-1-PHOSPHATE SYNTHASE FROM *LILIUM LONGIFLORUM* POLLEN, *NEUROSPORA CRASSA* AND BOVINE TESTIS

FURTHER EVIDENCE THAT A CLASSICAL ALDOLASE STEP IS NOT UTILIZED

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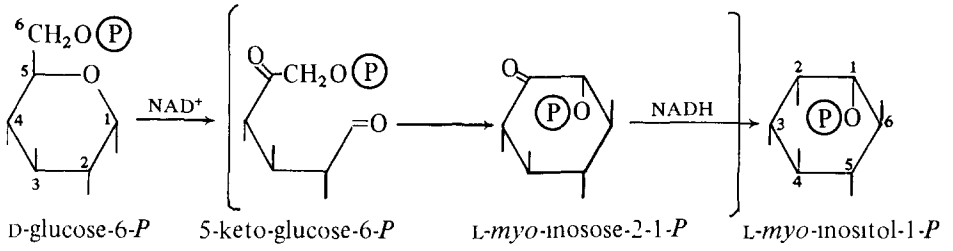
myo-Inositol-1-phosphate synthase (1L-*myo*-inositol-1-phosphate lyase (isomerizing), EC 5 5 1 4) preparations purified from the pollen of *Lilium longiflorum*, and from *Neurospora crassa* have been incubated with D-[5-^{18}O]-glucose-6-*P* and the *myo*-inositol which was formed was analyzed for retention of ^{18}O In each case, the isotope of oxygen was incorporated into the inositol without loss If a Schiff base had formed at the 5-position of the [^{18}O]glucose-6-*P*, the isotope should have been released to the incubation medium Supporting evidence that no Schiff base is formed at the 5-position, or at any other position, was obtained by incubation of the enzymes in media enriched with $H_2^{18}O$. If a Schiff base were formed and hydrolyzed, the regenerated carbonyl should become enriched to the level of the medium as reflected by the isotope content of the inositol product In no case did the product inositol have this degree of enrichment These data exclude consideration of a Class I aldolase mechanism for these enzymes The *Lilium* enzyme is unaffected by EDTA to a concentration of 100 mM The bovine enzyme is similarly uninhibited by EDTA to a concentration of 50 mM The *Neurospora* enzyme has previously been shown to be inhibited by these levels of EDTA and to be activated 2-fold by 2 mM Mg^{2+}, however, extensive dialysis against EDTA does not eliminate the metal independent activity of the enzyme In the present study, we have found that divalent metals show a range of stimulation/inhibition with the *Lilium* and the bovine enzymes, however, neither enzyme is dependent on the metals Thus, we suggest that these enzymes are not Class II aldolase enzymes either It is, therefore, possible that the *myo*-inositol-1-phosphate synthase from these species has an aldol step in the enzymatic pathway which is of neither classical aldolase type

Introduction

myo-Inositol-1-phosphate synthase (1L-*myo*-inositol-1-phosphate lyase (isomerizing), EC 5 5 1 4) catalyzes the cyclization of D-glucose-6-*P* using NAD^+ as a cofactor In the reaction C-1 and C-6 of D-glucose-6-*P* become covalently joined, C-6 of D-glucose-6-*P* becoming C-1 of L-*myo*-inositol-1-phosphate This condensation is thought to proceed by an aldol mechanism in which the intermediate 5-ketoglucose-6-*P* (D-xylo-hexos-5-ulose-6-*P*) is cyclized to L-*myo*-inosose-2 1-phosphate as shown in Scheme I

The aldolase reaction is thought to be of two general types Those described as Class I aldolases possess an intermediate step in which a Schiff base is formed between an amine group on the enzyme and a carbonyl group on the substrate or an enzymatically formed intermediate These enzymes can be detected by chemical reduction of the Schiff base, forming an inactivated enzyme with the substrate (intermediate) residue bound covalently They can also be detected



Scheme I

by the use of a substrate selectively labeled with ^{18}O at the site of formation of the Schiff base, the ^{18}O being lost in the reaction with the enzyme The latter experiment can be performed in reverse, in that ^{18}O from the incubation medium will be incorporated into the product of the enzyme reaction as a result of the hydrolysis of the Schiff base

The second type of classical aldolase is termed Class II These enzymes are thought to use divalent metals in the aldol transition state and can generally be shown to be dependent on such metals

Utilizing D-[5-^{18}O]glucose-6-*P*, which is a specific and unambiguous probe of Schiff base formation at C-5 of glucose-6-*P*[1], we find that *myo*-inositol-1-phosphate synthase from a *Lilium* and a *Neurospora* species do not use a Class I aldolase mechanism Experiments with $H_2{}^{18}O$ confirm that finding and also show that Schiff base formation does not occur at any other position of either the substrate or an intermediate Thus, these enzymes either utilize a Class II aldolase reaction, with the participation of a divalent metal, or the cyclization occurs by an atypical aldolase mechanism

Materials and Methods

Preparation of myo-inositol-1-phosphate synthase from L longiflorum pollen 10 g *L longiflorum* pollen var Ace was suspended in 60 ml 0 02 M Tris-acetate, pH 8 0/0 5 mM GSH Pollenkitt was removed by stirring with a glass rod, to which it sticks The suspension was homogenized with 50 strokes of a glass-glass homogenizer and the resulting mixture centrifuged for 0 5 h in a Sorvall rotor at 15 000 rev / min A 35–55% satd $(NH_4)_2SO_4$ ppt of this extract was dissolved in buffer and dialyzed for 4 h with two changes of buffer This was purified on a 2 × 30 cm DEAE-cellulose column, eluting with a 600 ml linear gradient of 0–0 25 M NaCl in the same buffer The fraction eluting between 0 145 and 0 195 M NaCl was concentrated by $(NH_4)_2SO_4$ (60% satd) precipitation and further purified on a 1 2 × 90 cm Sephadex G-200 column Pollen synthase elutes at 1 36 V_0 In the experiment in Table I, synthase purified through the Sephadex G-200 stage was used In the experiment of Table II, the enzyme was purified through the DEAE-cellulose step

Pollen enzyme assay *myo*-inositol-1-phosphate synthase was incubated at 30°C for 2 5 h in 0 5 ml 0 02 M Tris-acetate, pH 8 0/1 2 μmol D-glucose-6-*P*/ approx 0 5 μCi [1-^{14}C]glucose-6-*P*/0 5 mmol NAD/ 2 5 mmol ammonium acetate/Tris-EDTA or metal salt as specified The *myo*-inositol 1-phosphate produced was determined as described previously [2,3]

Preparation of myo-inositol-1-phosphate synthase from Neurospora crassa Neurospora crassa, strain RL 21 was cultured as described by Piña and Tatum [4] The mycelia were homogenized in a Waring Blendor and the supernatant fractionated with $(NH_4)_2SO_4$ and protamine sulfate as described elsewhere [4,5]

Purification of the enzyme to homogeneity was performed according to a chromatographic procedure (unpublished data) consisting of the following steps Gel filtration on Ultrogel AcA-34 (LKB), anionic exchange chromatography on DEAE-cellulose (Whatman DE-52), anionic exchange chromatography on AH-Sepharose 4B (Pharmacia) and adsorption chromatograpyy on calcium phosphate deposited on fibrous cellulose The enzyme obtained by this procedure is homogenous according to the following criteria analytical gel filtration, sedimentation in a sucrose density gradient, analytical discontinuous acrylamide gel electrophoresis, gel electrofocusing and SDS-polyacrylamide gel electrophoresis The

enzyme was assayed as described previously [5]

Preparation of myo-inositol-1-phosphate synthase from bovine testis The enzyme used in these experiments was purified by $(NH_4)_2SO_4$ precipitation on Celite-545 followed by reverse $(NH_4)_2SO_4$ gradient elution The Celite fraction containing activity was then purified on DEAE cellulose (Whatman DE-52) The enzyme purification and assay procedures were as described previously [1,6]

Analysis of the ^{18}O content of substrate and product Lyophilized incubation mixtures were shaken overnight with a solution of butaneboronic acid in pyridine (44 mg/ml) This produces *myo*-inositol 1,2 3,5 4,6-tris(butaneboronate) [7] and a bis-butaneboronate of glucose The samples were then divided in two and the portion to be analyzed for glucose was treated with 10 μl acetic anhydride to form α-D-glucofuranose cyclic 1,2 3,5-bis(butaneboronate) 6-acetate [8] Because the acetic anhydride causes some inositol boronate to be lost, the non-acetylated preparation was used to directly analyze inositol and the acetylated mixture was used to analyze glucose The ^{18}O content of the glucose and *myo*-inositol was measured as previously described [1] using electron ionization GC-MS In each case, the ions measured, using selected ion monitoring, were $[M-C_4H_9]^+$ and the ^{18}O-labeled ion 2 daltons heavier, i e , *m/z* 297 and 299 for the glucose derivative, *m/z* 321 and 323 for inositol and *m/z* 323 and 325 for sorbitol

D-[5-^{18}O]glucose-6-*P* was synthesized as previously described [1]

Results and Discussion

Although the intermediate in the *myo*-inositol-1-phosphate synthase reaction, 5-ketoglucose-6-*P* of Scheme I, has not been directly demonstrated there is much evidence supporting its formation The NAD^+ dependence of all known *myo*-inositol-1-phosphate synthases and the effect on the rate of the synthesis reactions, by deuterium and tritium substitution at C-5 of glucose-6-*P*, represent some of the evidence for the oxidation of the 5-hydroxyl of glucose-6-*P* (for a summary of these and other studies, see Refs 1 and 22) Supporting evidence for the intermediate is found in the facile base-catalyzed cyclization of 5-keto-glucose-6-*P* to inosose phosphates which can be reduced to inositol phosphates [9,10] Thus, if this reaction is of the Class I category, the 5-carbonyl is the expected site of Schiff base formation, and labeling this function with ^{18}O gives a specific probe of any covalent interaction between the 5-position of the intermediate and the enzyme Table I shows that

TABLE I

INCUBATIONS OF *myo*-INOSITOL-1-PHOSPHATE SYNTHASE WITH D-[5-^{18}O]GLUCOSE 6-PHOSPHATE

Incubations of enzyme and boiled enzyme controls with labeled substrate were followed by treatment with alkaline phosphatase to produce free D-glucose from the substrate and free *myo*-inositol from the product The samples were then derivatized and analyzed by GC-MS Enzyme from *Lilium* pollen was incubated for 24 h at 37°C in 0 5 ml of 40 mM Tris-acetate, pH 8 0, which was 2 9 mM in D-[5-^{18}O]glucose-6-*P*, 5 mM in ammonium acetate, 1 mM in NAD^+ and which contained 0 02% NaN_3 The initial rate of synthesis was 9 2 nmol *myo*-inositol 1-phosphate per h The reaction was terminated by heating at 100°C for 5 min This was followed by treatment with alkaline phosphatase (2 h, 37°C) Samples were then taken to dryness and analyzed The *Neurospora* preparations of *myo*-inositol-1-phosphate synthase were incubated for 24 h at 37°C with 0 15 ml 50 mM Tris-HCl, pH 7 7, which was 1 3 mM in D-[5-^{18}O]glucose-6-*P*, 2mM in NAD^+, 14 mM in NH_4Cl and which contained 0 02% NaN_3 The initial rate of synthesis of *myo*-inositol 1-phosphate with the DEAE-purified enzyme was 1 3 μmol/h and with the calcium phosphate-purified enzyme, 3 1 μmol/h In this case, the reaction was terminated by incubation with alkaline phosphatase for 1 h

Enzyme Source	Percent excess ^{18}O		Percent retention of ^{18}O in *myo*-inositol
	D-[5-^{18}O] glucose	[2-^{18}O] *myo*-Inositol	
L. longiflorum pollen	33 3 ± 0 6 [a]	34 5 ± 0 6 [b]	104
N crassa (DEAE)	34 ± 0 4 [d]	34 5 ± 0 3 [c]	101
(Calcium Phosphate)		34 6 ± 0 04 [c]	101

[a] Average ± S D of five values of 5-^{18}O]glucose were not enzymatically treated

[b] Average ± S D of three experimental samples

[c] Average ± S D of 12 samples from inactive enzyme incubations (inactivated at 100°C) and active enzyme incubations (these groups were not significantly different)

[d] Average ± S D of four samples

TABLE II

INCUBATIONS OF *myo*-INOSITOL-1-PHOSPHATE SYNTHASE IN BUFFERED $H_2{}^{18}O$

Samples were incubated with D-glucose-6-*P* in an $H_2{}^{18}O$-containing medium, followed by treatment with alkaline phosphatase Products were then analyzed by GC–MS as glucose or *myo*-inositol (*Lilium*) or following treatment with sodium borohydride (*Neurospora*) when sorbitol and *myo*-inositol were analyzed Enzyme from *Lilium* pollen was incubated for 24 h at 37°C in 30 mM Tris-acetate, pH 8 0, which was enriched in ^{18}O by dilution with $H_2{}^{18}O$ The medium was 2 4 mM in D-glucose-6-*P*, 1 mM in NAD^+ and 5 mM in ammonium acetate The inositol rate of synthesis was 3 nmol *myo*-inositol 1-phosphate per h The incubation mixture was then dried and the residue taken up in 0 5 ml of H_2O, alkaline phosphatase added and the mixture incubated a further 2 5 h, heated at 100°C, and the protein removed by centrifugation The supernatant was lyophilized and derivatized for GC–MS The *Neurospora* enzyme was incubated for 24 h in an ^{18}O-enriched Tris-HCl medium, pH 7 7/D-glucose-6-*P* (1 mM)/ 2 mM $NAD/(NH_4)_2SO_4$ (25 mM)/0 02% NaN_3 The initial rate of synthesis was 4 μmol/h Alkaline phosphatase treatment was followed by reduction with 160 mg $NaBH_4$ The resulting mixture was lyophilized and the sorbitol and *myo*-inositol were derivatized and analyzed by GC–MS

Enzyme Source	^{18}O enrichment of incubation medium	Percent isotope incorporation [a]		
		Control	Substrate	*myo*-Inositol product
		Glucose		
L longiflorum pollen	7 3%	0 28 [b]	2 6 ± 2 6 [c]	1 6 ± 0 1 [c]
		Sorbitol		
N crassa	18 8%	13 9 ± 1 5 [d]	13 4 ± 1 4 [d]	14 4 ± 1 3 [e]

[a] Percent molecules containing one atom of ^{18}O calculated as follows For glucose as the bis(butaneboronate) acetate derivative, *m/z* 297 contains all the oxygen atoms of glucose (it is the derivative less a butyl radical [8]) Enrichment by a single ^{18}O atom is thus measured at *m/z* 299 The percent abundance of *m/z* 299 in the enriched molecule, less the percent abundance of *m/z* 299 in the naturally-occurring molecule is the percent isotope incorporated For inositol the corresponding ions are *m/z* 321 and 323, for sorbitol *m/z* 323 and 325

[b] Enzyme (n = 2) was heated (100°C) to inactivate it prior to adding substrate

[c] Reaction stopped by heating, results are the average ± S D of three samples

[d] Average values ± S D Sorbitol in controls, n = 6, from active enzyme preparations, n = 3

[e] Average ± S D , n = 3

^{18}O is retained by *myo*-inositol formed in the reaction of this substrate with synthase from either the *Lilium* or the *Neurospora* species Thus, it is highly unlikely that the Class I aldolase mechanism is used by either of these enzymes *

Table II shows that, when either the lily pollen or the mold enzymes are incubated with D-glucose-6-*P* in a buffer containing $H_2{}^{18}O$, the inositol formed does not reach the level of isotope enrichment of the solvent If a Schiff base were formed between any position of the substrate and the enzyme, it would have to be hydrolyzed with water from the buffer and thus attain the same degree of labeling at the reacting carbon as the ^{18}O-content of the buffer The observed partial uptake of label from the medium is due to exchange of the aldehyde carbonyl of the glucose during mutarotation, a process which is accelerated at the pH of the incubation [11] **

* It is possible that the 5-carbonyl of 5-keto-glucose-6-*P* could react with an amine on the *myo*-inositol-1-phosphate synthase to form the aminol precursor of a Schiff base which might either, (1) dehydrate to give the Schiff base, but conserve the water for later hydrolysis or, (2) stabilize the aminol so that it did not dehydrate The latter instance would not, however, produce the enamine-imine tautomeric system which is thought to promote the proton removal, in this case from C-6, which precedes the aldol condensation The possibility that the water lost from the aminol is conserved cannot be excluded

** If, in the *Lilium* experiment, the reaction had used the Schiff base route the product 2-[$^{18}O_1$]*myo*-inositol

The cause of the variability in the amount of ^{18}O incorporated in these experiments is unknown but is similar to that which was observed with mammalian *myo*-inositol-1-phosphate synthase preparations [1]

Earlier studies on the *myo*-inositol-1-phosphate synthase from *Neurospora* agree with our findings, in that $NaBH_4$ only inhibited the enzyme at high concentrations and because the action of agents which inhibit Schiff base formation suggested that the enzyme was not a typical Class I aldolase [12] Whether the *Neurospora myo*-inositol-1-phosphate synthase is a Class II aldolase is uncertain The enzyme is 50% inhibited by 25 mM EDTA and completely inhibited with 100 mM EDTA [12] About 2-fold activation of the *Neurospora* enzyme occurs with 2 mM Mg^{2+} but the enzyme is active without the metal, even after a 24 h dialysis against 100 mM EDTA [13] Unless there is a metal which is very tightly bound, this suggests that the *Neurospora* enzyme has neither a Class I nor a Class II aldolase mechanism

Lily pollen synthase is not affected, positively or negatively, by Tris-EDTA up to 100 mM $MgCl_2$ inhibited at a concentration of 10 mM and above possibly due to precipitation of the enzyme $MnCl_2$ had no affect at concentrations of 0 2 and 2 mM Thus, the pollen synthase does not fit the definition of a Class II aldolase either

would be enriched to the level of the medium, 7 3% Since it is evident that the substrate takes up ^{18}O by exchange with the medium, presumably to form 1-[$^{18}O_1$]glucose-6-*P*, these molecules can form L-6-[$^{18}O_1$]*myo*-inositol to some extent (by hydrolysis of the Schiff base with $H_2{}^{16}O$) This would raise the percentage of $^{18}O_1$-*myo*-inositol molecules to even greater than 7 3% Since the observed amount is 1 6 ± 0 1% the Schiff base route is clearly excluded In the case of the *Neurospora* experiment the ^{18}O exchanged into glucose-6-*P* is trapped in sorbitol If no exchange into glucose-6-*P* occurred prior to the synthase reaction, and the reaction took the Schiff base route, 18 8% of the molecules measured would be 2-[$^{18}O_1$]*myo*-inositol, greater than the 14 4 ± 1 3% observed Since ^{18}O-uptake by glucose-6-*P* did occur during the incubation some of this would be incorporated into *myo*-inositol The rate of exchange relative to the synthase rate is not known but it is clear that some additional ^{18}O, would be taken up The maximum amount would be the sum of the 6-[$^{18}O_1$]- and 2-[$^{18}O_1$]*myo*-inositol molecules, calculated to be 27 3%

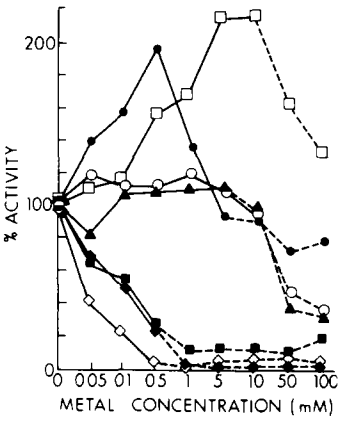


Fig 1 The effect of divalent metal ions on bovine testis *myo*-inositol-1-phosphate synthase purified through the DE-52 stage Incubations were carried out with $MgCl_2$ (□), $MnCl_2$ (●), $CoBr_2$ (○), $CaCl_2$ (▲), $CdCl_2$ (■), $Zn(C_2H_3O_2)_2$ (♦), $Cu(C_2H_3O_2)_2$ (◇) for 1 h and analyzed colorimetrically Concentrations where the enzyme appeared to precipitate are indicated by a broken line

We find with bovine synthase that potassium-EDTA is 2 7-fold stimulatory at 5 mM but this must be due to activation by K^+, which has been reported with this enzyme [6] When Tris-EDTA is incubated with the bovine synthase there is no effect to a concentration of 10 mM followed by a 1 5-fold stimulation at 50 mM Rat testis *myo*-inositol-1-phosphate synthase is similarly unaffected by EDTA [14] This suggests, but does not prove, that there is no divalent metal ion associated with either the bovine enzyme or that from rat testis

Mg^{2+} activation of bovine testis *myo*-inositol-1-phosphate synthase occurs up to concentrations of 10 mM (Fig 1) A similar stimulation occurs with Mn^{2+} Other metal ions which could be considered to be candidates for Class II aldolase activation were found to be inactive (Co^{2+} and Ca^{2+}) or inhibitory (Cd^{2+}, Cu^{2+} and Zn^{2+}) It is thus possible that the *Neurospora myo*inositol-1-phosphate synthase and that from bovine testis are both being affected in a similar way, stimulated by, but not dependent on, certain divalent cations

There is some controversy over the mechanism of the aldolase step in the *myo*-inositol-1-phosphate synthase from rat testis Pittner and Hoffmann-Ostenhof [15] have reported that glucose-6-*P* can be

linked covalently to the enzyme following $NaBH_4$ treatment of an incubation mixture The authors report that the glucose-6-*P* is joined to a lysine residue through C-1 of the glucose [16] Work from another laboratory [14] suggests that this is an artifact, the result of an adventitous reaction between glucose and lysine residue on a protein which co-purifies with rat testis synthase Furthermore, on purely theoretical grounds, the formation of a Schiff base between D-glucose-6-*P* and the synthase at C-1 does not produce an intermediate which would facilitate the aldol reaction in this manner suggested by Rutter [17]

The *myo*-inositol-1-phosphate synthase from *Lemna gibba* is reported to be completely inhibited by 10 mM EDTA, the inhibition being reversed by Mn^{2+} but not by Mg^{2+} [18] Another *myo*-inositol-1-phosphate synthase, from *Streptomyces griseus* is inhibited by 60 mM EDTA, and this inhibition is reversed by Mg^{2+} [19] These enzymes thus may be Class II aldolases of the classical variety, with clear divalent metal dependence

Both bovine and rat testis *myo*-inositol-1-phosphate synthase have been found to be stimulated by K^+ and it has been proposed that this activation is due to stabilization of the carbanion generated in the step prior to C1-C6 condensation [1] Since this is the formal function of the Schiff base and of the divalent metal in classical aldolases, perhaps the enzymes from rat, ox, *Neurospora* and *Lilium* have a previously undescribed mechanism of action which is facilitated by certain monovalent cations such as NH_4^+ and K^+ but which is also functional in their absence In support of this it is known that both 5-ketoglucose [20] and 5-ketoglucose-6-*P* [9,10] are readily cyclized by dilute base to form inososes which can be reduced to inositols The occurrence together, in one molecule, of an aldehyde (C-1) and an activated methylene (C-6) may be sufficient for the C1-C6 condensation to require no further stabilization In this case, the enzyme would function as an oxidoreductase, as a basic catalyst and to provide the stereospecificity necessary to produce only the *myo*-inositol isomer

Perhaps the classical categories of aldolases types are too limiting It is becoming evident that aldolases do not follow strict phylogenetic lines with respect to mechanism Using [2-^{18}O]Fru-P_2 as a substrate to detect Schiff base formation in Fru-P_2 aldolases from several sources, Heron and Caprioli showed *Euglena gracilis* to simultaneously elaborate both Class I and Class II enzymes [21] They also found that the Fru-P_2 aldolase of *L casei* formed a Schiff base, thus showing that bacteria as well as higher organisms can elaborate a Class I aldolase Whether the *myo*-inositol-1-phosphate synthases we have studied utilize another type of mechanism or have a tightly bound metal (thus, being a special type of Class II enzyme) has not been established However, the possibility that the Class I and Class II mechanisms are not sufficient for all cases should be considered in the study of enzymes using the aldolase reaction

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