BBA 66753

BEHAVIOR OF CYCLOALDOLASE FROM *NEUROSPORA CRASSA* TOWARDS SUBSTRATE ANALOGS AND ALDOLASE INHIBITORS

MIGUEL MOGYOROS, AURORA BRUNNER AND ENRIQUE PIÑA

Departamento de Bioquímica, Facultad de Medicina, Universidad Nacional Autónoma de México, (Mexico)

(Received June 16th, 1972)

SUMMARY

Several characteristics of the D-glucose-6-phosphate: L-*myo*-inositol-1-phosphate cycloaldolase (NAD$^+$-dependent) from *Neurospora crassa* were investigated.

At a concentration of 5 mM, the following compounds inhibit the enzymatic reaction in decreasing order of effectiveness: D-sorbitol-6-*P*, D-ribose-5-*P*, D-fructose-6-*P*, 6-phospho-D-gluconate, 5-*keto*-D-gluconate and D-galactose-6-*P*. At similar concentrations D-glucosamine-6-*P*, D-glucose-6-sulphate, *myo*-inosose, D-glucose and D-mannose-6-*P* fail to inhibit the activity.

NADP$^+$ does not substitute for NAD$^+$ as coenzyme and NADPH is a very poor inhibitor compared to NADH.

The reaction is not inhibited by pyridoxal-5'-*P*, trinitrobenzenesulphonate or $NaBH_4$ which classically inhibit reactions where a Schiff base is formed between enzyme and substrate. The reaction was inhibited by high concentrations of EDTA.

INTRODUCTION

Some properties of an enzyme obtained from *Neurospora crassa* capable of forming L-*myo*-inositol-1-*P** from D-Glc-6-*P* called D-glucose-6-phosphate: L-*myo*-inositol-1-phosphate cycloaldolase** (NAD$^+$-dependent) (EC number not yet assigned) have already been studied[1]: the enzyme does not utilize D-Gal-6-*P*, D-Man-6-*P*, D-Fru-6-*P*, 6-phospho-D-gluconate and D-glucose as substrates. This suggested that the enzyme had a certain stereospecificity and several analogues of D-Glc-6-*P* were studied in order to find out whether they serve as substrates or whether they act as inhibitors of the reaction and thus some of the stereospecificity characteristics of the enzyme could be ascertained. Furthermore, it has also been observed that the cofactor for this enzyme is NAD$^+$ (refs 1 and 2) and that several NAD$^+$ analogues

* Inositol is used as an abbreviation for *myo*-inositol.

** Cycloaldolase is used as an abbreviation for D-glucose-6-phosphate: L-*myo*-inositol-1-phosphate cycloaldolase.

were unable to substitute for it in the reaction[1]. The study on the specificity for this coenzyme was extended to $NADP^+$ when it was possible to separate the cycloaldolase from glucose-6-phosphate dehydrogenase (EC 1.1.1.49).

On the other hand, aldolases have been divided into two groups: I and II[3]. Type I aldolases fix their substrate by forming a Schiff base[4] and Type II aldolases have a metal requirement[5]. Neither of these two mechanisms has been investigated in cycloaldolase, so some experiments were conducted in order to investigate whether one of these mechanisms is involved in cycloaldolase activity.

MATERIALS AND METHODS

Chemicals

myo- or *scyllo*-inosose was prepared by the method of Posternak[6]. H_2SO_4, ascorbic acid, KH_2PO_4, D-glucose, $(NH_4)_2SO_4$, NH_4Cl and $NaIO_4$ were obtained from E. Merck AG., Darmstadt. Ammonium heptamolybdate and trichloroacetic acid were purchased from Allied Chemical Corporation. Protamine sulphate obtained from Nutritional Biochemicals Corporation; sodium sulphite from Reasol (Mexico); acrylamide, *N,N'*-methylenbisacrylamide and trinitrobenzenesulphonate from Eastman Kodak Corporation; D-[6-^{3}H]glucose-6-*P* from International Chemical and Nuclear Corporation; all other substances were acquired from Sigma Chemical Company.

Enzyme

The enzyme was prepared with slight modification of the method already described[7]. For most of the experiments the enzyme was purified approximately 300-fold, for some experiments a preparation with a 10-fold higher specific activity was used. This purification involved an acrylamide gel step.

Methods

The enzymatic activity was determined by three different methods. The procedure chosen depended on which was more suitable for the experiment.

In the biological assay, the product of the enzymatic reaction, inositol-1-*P*, was hydrolyzed with alkaline phosphatase (EC 3.1.3.1) and free inositol was measured according to McKibbin[8]. Also the spectrophotometric assay described by Barnett *et al.*[9] was employed. A radioactive assay based on the liberation of one ^{3}H from D-[6-^{3}H]glucose-6-*P* during the synthesis of inositol-1-*P*[10], was also employed.

RESULTS AND DISCUSSION

Inhibition of the enzyme by carbohydrates with similar structure to the substrate

In order to study some steric requirements of the active center in cycloaldolase, various compounds similar in their structure to D-Glc-6-*P* were tested as possible inhibitors of the system. Their inhibitory effects are shown in Table I. According to the results the following conclusions may be drawn: (1) Only the carbohydrates that have a phosphate residue inhibit cycloaldolase; however, not all the phosphorylated

TABLE I

INHIBITION OF CYCLOALDOLASE BY COMPOUNDS SIMILAR TO THE NATURAL SUBSTRATE

The incubation mixture contained in 1 ml of final volume: 100 μmoles of Tris–HCl buffer (pH 7.7), 14 μmoles of NH_4Cl, 1 μmole of NAD^+ and cycloaldolase purified approximately 300-fold (or 3000-fold in those instances in which it was used after the acrylamide gel step). The samples were incubated for 1 h at 30 °C. The inhibitors were added at the final concentrations indicated in the table, keeping the final volume to 1.0 ml. The enzymatic activity was measured by one of the following methods: S, spectrophotometric, M, microbiological or R, radioactive and according to the details given in Materials and Methods.

Compound	*% inhibition when incubated with 10 mM* D-*Glc-6-P*			*Method*
Concn:	*5 mM*	*10 mM*	*15 mM*	
D-Sorbitol-6-*P*	66	93	98	S
D-Rib-5-*P*	50.7	69.8	95.2	M
D-Fru-6-*P**	47.5	84.8	90	M
6-Phospho-D-gluconate	23	40	57	S
5-*keto*-D-Gluconate	7	10	14	S
D-Gal-6-*P*	3.7	9.6	13.3	M
D-Glucosamine-6-*P*	0	0	0	M
D-Glucose-6-sulphate	0	0	0	S
myo-Inosose	0	0	0	S
Concn:	*50 mM*	*100 mM*	*150 mM*	
D-Man-6-*P**	25.5	34.2	54.3	R
D-Glucose	9	83	93	S

* Measured after submitting the enzymatic preparation to electrophoresis through an acrylamide-gel column.

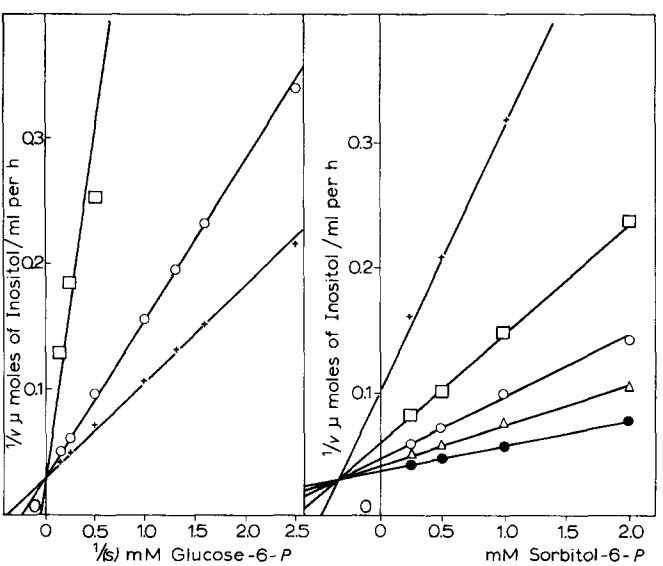

Fig. 1. Left. Lineweaver–Burk plot for cycloaldolase activity. Without inhibitor (×—×); with $2.5 \cdot 10^{-6}$ M 6-phospho-D-gluconate (○—○) and with $2 \cdot 10^{-6}$ M D-sorbitol-6-*P* (□—□). Experimental conditions as in Table I. Right. Graphical method to evaluate the inhibition constant of D-sorbitol-6-*P*. Five concentrationes were used (M): $1 \cdot 10^{-6}$ (●—●); $2 \cdot 10^{-6}$ (△—△); $4 \cdot 10^{-6}$ (○—○); $6 \cdot 10^{-6}$ (□—□) and $8 \cdot 10^{-6}$ (×—×). The rest of the incubation mixture was the same as in Table I. The enzymatic activity was measured by the spectrophotometric method.

compounds were inhibitory. D-Sorbitol-6-*P*, D-Rib-5-*P*, D-Fru-6-*P* and 6-phospho-D-gluconate were effective inhibitors, while D-Man-6-*P* exerted a significant inhibitory effect but at a concentration 10 times higher than the former compounds. D-Gal-6-*P* and D-glucosamine-6-*P* were practically without effect. Of certain interest is that D-glucose-6-sulfate had no effect. (2) All the compounds that exert an inhibitory action may exist in an open form, and those that exist exclusively in open form as D-sorbitol-6-*P* and 6-phospho-D-gluconate, induce a very significant inhibition. In this respect, it has been postulated that the enzyme acts on the open form of D-Glc-6-*P*. As can be seen in Fig. 1 (left) D-sorbitol-6-*P* and 6-phospho-D-gluconate give competitive inhibition with an apparent K_i for D-sorbitol-6-*P* of $3.4 \cdot 10^{-4}$ M (Fig. 1, right). (3) D-Glc-6-*P* could be displaced from the active center of the enzyme when the carbonyl group of glucose was replaced by an alcohol as in D-sorbitol-6-*P* as well as by a carboxyl group as in 6-phospho-D-gluconate, but according to the data the alcohol group is more effective. (4) The orientation and substitution of the OH residue of carbons 2, 4 and 5 seems to be critical in order to inhibit the enzyme. Effective competitive inhibitors are those having their OH radicals of these three carbons oriented in the same position as D-glucose (observe the better inhibition obtained with D-sorbitol-6-*P* and 6-phospho-D-gluconate as compared to the lower inhibition detected with D-Gal-6-*P* and D-Man-6-*P*). (5) In addition to the orientation of carbons 4 and 5 in a hexose, or 3 and 4 in a pentose, the compounds retain their inhibitory ability when the OH in carbon 2 in a hexose, or carbon 1 in a pentose, was substituted by a carbonyl group as in D-Fru-6-*P* or D-Rib-5-*P*, respectively. But if this OH was replaced by an amino group, as in D-glucosamine-6-*P* the resulting substance was neither an inhibitor nor a substrate (see below).

Substitution of substrate for other monosaccharides

In order to test the possibility that the compounds listed in Table I could be used as substrate for the cycloaldolase, each one of these substances was incubated at 30 °C for 1 h with the complete incubation mixture except that D-Glc-6-*P* was omitted. Under these conditions cycloaldolase was unable to use the compounds listed in this table as substrates by the following criteria: (a) with the phosphorylated and the nonphosphorylated compounds assayed by the microbiological method, cycloaldolase and phosphatase did not form a substance capable of substituting for inositol in the biological assay described by McKibbin[8]. (b) With the phosphorylated compounds studied by the spectrophotometric technique, cycloaldolase never produced a substance capable of liberating P_i, as inositol-1-*P* does when subject to the assay conditions described by Barnett *et al.*[9]. However, the possibility that one of the substances could be used by the enzyme giving a different product from the ones we studied cannot be dismissed, specially if one takes into consideration that D-Man-6-*P* is transformed into *neo*-inositol[11].

Effect of inorganic phosphate on cycloaldolase activity

It has been observed that P_i inhibits cycloaldolase[1], but since these experiments were performed with the biological assay which requires alkaline phosphatase in order to obtain inositol from inositol-1-*P*, it is possible that P_i could inhibit the phosphatase reaction and not the cycloaldolase reaction. However, when the activity was tested by the radioactive assay, in which the step with alkaline phosphatase is

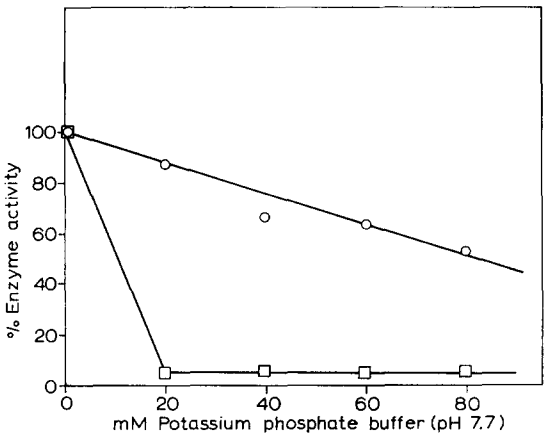

Fig. 2. Effect of phosphate buffer on the cycloaldolase activity. Two sets of tubes were prepared as follows: potassium phosphate buffer (pH 7.7) at the concentrations shown in the figure, 1 μmole of NAD^+, 14 μmoles of NH_4Cl and cycloaldolase (final volume 0.9 ml). 10 μmoles of D-Glc-6-*P* were immediately added to one set of tubes (○—○) and the enzymatic reaction was followed for 1 h at 28 °C. The other set was preincubated for 1 h at 28 °C (□—□) in the absence of D-Glc-6-*P* which was added at the end of this hour, continuing the incubation for 1 h more in order to measure the enzymatic activity. The product formed by the enzymatic reaction was evaluated by the radioactive method.

omitted the inhibition was again observed (Fig. 2). In the same figure it is interesting to note that if the enzyme was preincubated with P_i for 1 h, the inhibition was total, suggesting that P_i may react with the enzyme changing its structure or blocking the active center, and that this reaction needs a certain time to take place.

Studies on cycloaldolase coenzyme

NAD^+ is the natural cofactor for cycloaldolase and according to the data presented in a previous paper[1], its specificity is absolute. When it became possible

TABLE II

EFFECT OF SEVERAL PYRIDINE NUCLEOTIDES ON THE ACTIVITY OF CYCLOALDOLASE

The incubation mixture and the experimental conditions are identical to those described for Table I except for NAD^+ which was added, where indicated, at the final concentrations shown in the table. The enzymatic activity was measured by the spectrophotometric method. The radioactive method was used when the purer enzyme was employed.

Concn of nucleotide (mM)	*% of enzyme activity*
NAD^+ (1)	100
NADH (1)	0
$NADP^+$ (1)*	0
NADPH (1)	0
NAD^+ (1) + $NADP^+$ (0.3)*	100
NAD^+ (1) + $NADP^+$ (1)*	100
NAD^+ (1) + $NADP^+$ (3)*	100
NAD^+ (1) + NADPH (1)	98.3
NAD^+ (1) + NADPH (5)	96.5
NAD^+ (1) + NADPH (10)	88.5

* This experiment was done after subjecting the enzymatic preparation to electrophoresis through an acrylamide-gel column.

to separate cycloaldolase from glucose-6-*P* dehydrogenase in an acrylamide gel column, the experiments on the specificity of the cofactor could be extended to $NADP^+$, which did not function as coenzyme (Table II), NADPH was a very poor inhibitor (Table II). On the other hand, NADH is a competitive inhibitor with an apparent K_i of $6.8 \cdot 10^{-5}$ M (Fig. 3). This value, obtained with the spectrophotometric assay, is slightly different from the one previously obtained with the biological assay[1].

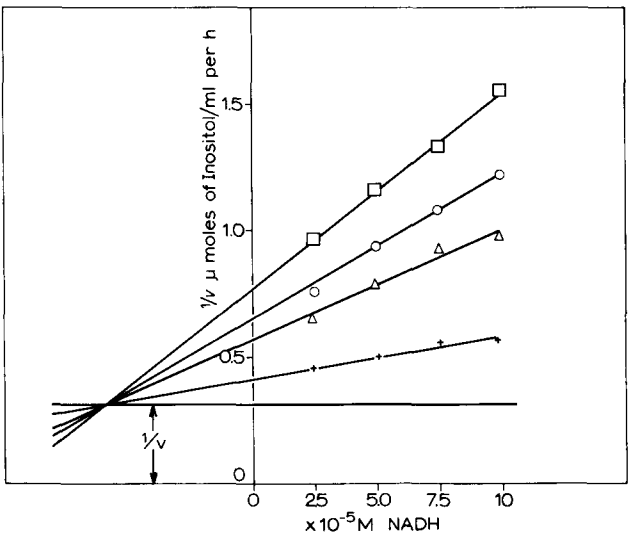

Fig. 3. Graphical method to evaluate the inhibition constant of NADH in the cycloaldolase reaction. Four concentrations of NAD^+ were assayed (M): $1 \cdot 10^{-5}$ (×—×); $2.5 \cdot 10^{-5}$ (△—△); $5 \cdot 10^{-5}$ (○—○); and $7.5 \cdot 10^{-5}$ (□—□). The other experimental conditions as in Table I. The enzymatic activity was detected by the spectrophotometric method.

Behavior of cycloaldolase toward compounds that inhibit a Schiff base formation

The ε-amino groups of lysines are involved in the reaction of Type I aldolases[4] and thus they are inhibited by $NaBH_4$ (ref. 12) and pyridoxal-5'-*P* (ref. 13) because of Schiff base formation and should also be inhibited by trinitrobenzenesulphonate[14]. $NaBH_4$ produced an inhibition of cycloaldolase (Table III) under conditions where it usually abolishes the activity of other aldolases[12]. The data in Table III indicate that the enzyme was not affected by trinitrobenzenesulphonate. Pyridoxal-5'-*P* inhibits at high concentrations but so does pyridoxamine-5'-*P* which should not form a Schiff base, this suggests that the inhibition is a side effect most probably due to high concentration of these substances and not to an inhibition of the formation of a probable Schiff base.

Cycloaldolase as a metallo-aldolase?

Type II aldolases or metallo-aldolases are strongly inhibited by EDTA[3]. With cycloaldolase we found the same response (Fig. 4). This fact, together with other properties such as: an enhancement in the activity due to NH_4^+ (ref. 1), a very limited pH range in the enzymatic activity[15] and the fact that fungi are organisms where they have been found[3], tend to support the possibility that D-glucose-6-*P* cycloaldolase from *N. crassa* belongs to the Type II aldolases.

TABLE III

EFFECT OF COMPOUNDS THAT INHIBIT TYPE I ALDOLASES ON THE CYCLOALDOLASE REACTION

For pyridoxal-5'-*P* and pyridoxamine-5'-*P*, the experimental conditions and incubation mixture were as indicated for Table I, except that D-Glc-6-*P* was used at the final concentrations shown in the table. The enzymatic activity was measured by the radioactive method. The trinitrobenzenesulphonate adjusted at pH 7.7 with NaOH was preincubated with the enzyme for 1 h at 30 °C in the absence of all other components of the incubation mixture. This was followed by the addition of the incubation media with the concentrations of D-Glc-6-*P* indicated. The enzymatic activity was measured by the radioactive technique. In the study with $NaBH_4$ the experiments were performed as follows: to two separate tubes, one with the complete experimental mixture (Table I) and the other one identical but without D-Glc-6-*P*, were added ten successive alternate aliquots of 0.106 M $NaBH_4$ and of 1 M acetic acid to keep the pH around 6.0 in the conditions described by Horecker[12]. This was followed by exhaustive dialysis. The enzymatic reaction was measured by the microbiological technique. The enzymatic activity recorded in the tube that received $NaBH_4$ without D-Glc-6-*P* was taken as 100% of the activity.

Compound and final concn (mM)	*% inhibition when incubated with 1 mM D-Glc-6-P*
Pyridoxal-5'-*P* (1)	20
Pyridoxal-5'-*P* (10)	91.15
Pyridoxamine-5'-*P* (1)	10.1
Pyridoxamine-5'-*P* (10)	59.5
	% inhibition when incubated with 10 mM D-glucose-6-P
Pyridoxal-5'-*P* (10)	87.5
Pyridoxamine-5'-*P* (10)	67.3
Trinitrobenzenesulphonate (10), (20), (30) or (40)	0
$NaBH_4$ (106)	43.5

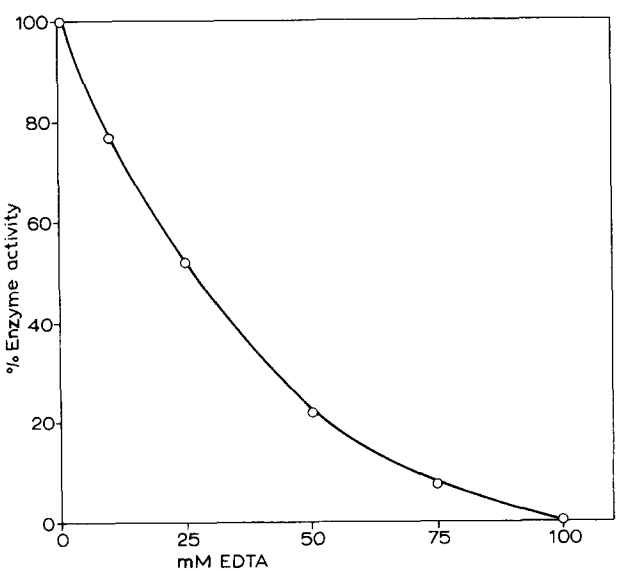

Fig. 4. Effect of EDTA on the cycloaldolase activity. Experimental conditions as in Table I. The chelating agent was added at the final concentrations indicated in the figure. The cycloaldolase activity was assayed by the radioactive technique.

ACKNOWLEDGEMENTS

The authors want to thank Dr Armando Gómez-Puyou for his help in the preparation of the manuscript.

REFERENCES

1 E. Piña, Y. Saldaña, A. Brunner and V. Chagoya, *Ann. N.Y. Acad. Sci.*, 165 (1969) 541.
2 A. Brunner, M. Z. Piña, V. Chagoya and E. Piña, *Arch. Biochem. Biophys.*, 150 (1972) 32.
3 W. J. Rutter, *Fed. Proc.*, 23 (1964) 1248.
4 E. Grazi, P. T. Rowley, T. Cheng, O. Tchola and B. L. Horecker, *Biochem. Biophys. Res. Commun.*, 9 (1962) 38.
5 O. Warburg and W. Christian, *Biochem. Z.*, 314 (1943) 149.
6 Th. Posternak, *Biochem. Prep.*, 2 (1954) 57.
7 E. Piña and E. L. Tatum, *Biochim. Biophys. Acta*, 136 (1967) 265.
8 J. M. McKibbin, *Methods Biochem. Anal.*, 7 (1957) 120.
9 J. E. Barnett, E. E. Brice and D. L. Corina, *Biochem. J.*, 119 (1970) 183.
10 I. W. Chen and F. Charalampous, *Biochim. Biophys. Acta*, 136 (1967) 568.
11 W. R. Sherman, S. L. Goodwin and K. D. Gunnell, *Biochemistry*, 10 (1971) 3491.
12 B. L. Horecker, S. Pontremoli, C. Ricci and T. Cheng, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 1949.
13 M. Rippa and L. S. Pontremoli, *Arch. Biochem. Biophys.*, 118 (1967) 48.
14 C. J. Coffee, R. A. Bradshaw, C. R. Goldin and C. Frieden, *Biochemistry*, 10 (1971) 3516.
15 A. Brunner, *Purificación y propiedades de la* D-*glucosa-6-P:* L-*mio-inositol-1-P cicloaldolasa de Neurospora crassa*, Thesis, Facultad de Química, UNAM, México, 1972.