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Inhibition by different amino acids of the aspartate kinase and the homoserine kinase of the yeast *Saccharomyces cerevisiae*

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In this paper, we describe a simple method to measure the yeast homoserine kinase and aspartate kinase activities, independently but in the same extract. With this method, we have determined some kinetic parameters for the physiological substrates of both enzymes, and investigated the inhibition exerted by different amino acids on these activities. Of all natural amino acids tested, only threonine inhibits effectively both enzymatic activities, although to a different degree. We did not find the reported inhibition by L-homoserine over the aspartate kinase. Altogether the data point to the aspartate kinase and to the threonine as the key factors in the regulation of this route.

Aspartate kinase; Homoserine kinase; Threonine biosynthesis; *Saccharomyces cerevisiae*

1. INTRODUCTION

In bacteria and fungi, threonine and methionine are synthesized from aspartate through a common metabolic sequence which leads to homoserine in three steps. From that point on, the pathways for methionine and threonine diverge. In bacteria and in certain fungi but not in yeast, lysine also is derived from aspartate.

The regulation of the threonine/methionine pathway occurs at two different levels: the regulation of enzyme synthesis by control of gene expression and the regulation of enzyme activity controlling the flow of metabolites through the pathway. As usually happens with most pathways, not all enzymes and genes involved in the synthesis of these amino acids are equally controlled. In fact, in the yeast *Saccharomyces cerevisiae*, the regulation takes place mainly at the first steps, both of the common part of the route which is dependent on the aspartate kinase (ASK) activity, and of the specific branches for threonine and methionine catalyzed by the homoserine kinase (HSK) and the homoserine acetyltransferase, respectively (for a review, see [1]).

The ASK activity converts aspartate to β-aspartylphosphate and it is absent in *hom3* mutants; it is controlled by threonine both through repression and feedback-inhibition. It has been reported that homoserine, methionine and lysine also exert certain regulation over this activity [2]. Gene *HOM3* has been recently cloned [3].

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The HSK converts homoserine to *O*-phosphohomoserine. It is determined by gene *THR1*, which has also been cloned [4]. The final product, threonine, regulates the HSK enzymatic activity, while methionine seems to control its synthesis [5].

Our main goal with this work has been to try to establish the relative importance of the ASK and the HSK steps in the control of the metabolic flow that leads to threonine production by a yeast cell. In this light, we have set up a rather simple and reliable method to purify and assay both enzymes. We have also determined some kinetic parameters of the enzymes and studied the effect of different amino acids on their activities.

2. MATERIALS AND METHODS

2.1. *Strains*

Strains X2180-1A (*MATa SUC2 mal gal2 CUP1*) and D160-2C (*MATα ilv1 ura3 hom3 his1 arg6 trp2 ade1 met1 gal2*) were provided by the Yeast Genetic Stock Center (Berkeley, USA); strain F15 (*MATα thr1 arg4*) was provided by La Cruz del Campo SA (Sevilla, Spain).

2.2. *Media*

Usual minimal medium (SD) was used throughout this work; when necessary, it was supplemented with the appropriate requirements [6].

2.3. *Enzymes and chemicals*

Lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40) from rabbit muscle and phosphoenolpyruvate (tricyclohexyl ammonium salt), were from Sigma; L-homoserine was obtained from Fluka; α-amino-β-hydroxyvaleric acid (hydroxynorvaline) was a gift from Degussa ABM (Germany) and it contains the four diastereoisomers; all other biochemicals were purchased from Sigma. Inorganic compounds were from Merck.

2.4. *Buffer solutions*

AT buffer [7] contains 40 mM phosphate (pH 7.2) with 0.1 M KCl_2, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM DTE and 1 mM L-threonine; BT buffer is identical to AT except that the concentration of phosphate was 20 mM.

2.5. *Partial purification of the enzymes*

Aspartate kinase and homoserine kinase were purified by a procedure substantially modified from that described by Thèze et al. [8] for *Escherichia coli*. Yeast cells were grown with a vigorous stirring, at 30°C in minimal (SD) medium supplemented with the appropriate amino acids. When the culture reached an OD_{600} of 0.7 (late exponential phase), the cells were harvested by centrifugation at room temperature, washed twice and resuspended in AT buffer and disrupted in a Braun homogenizer with 0.5 mm diameter glass beads. Cell debris was removed by two consecutive centrifugations at 4°C, one of 20 min at 8000 × *g*, and the last one of 2 h, at 105000 × *g*.

Protamine sulphate (5 mg/g wet weight) neutralized to pH 7 with NaOH, was added to the crude extract and, after 1 h stirring at 4°C, the precipitate was removed by centrifugation in the conditions described above. Ammonium sulphate was added in order to bring the supernatant to 30% saturation, and the mixture stirred for 30 min at 4°C. The precipitate (fraction P30) was separated by centrifugation, and the supernatant was brought to 45% $(NH_4)_2SO_4$ saturation. The pellet (fraction P45) obtained after a 30 min incubation at 4°C and centrifugation, was dissolved in buffer BT, dialyzed extensively against the same buffer and stored at −80°C. Under these conditions no detectable loss of activity over a period of 1 month was observed.

2.6. *Enzyme assays*

2.6.1. Assay of HSK

Homoserine kinase activity was routinely assayed by monitoring ADP production using the pyruvate kinase-lactate dehydrogenase coupled assay [8,9]. One unit of activity (U) was defined as the amount of enzyme required to convert 1 μmol of ATP to ADP per minute at 30°C. The standard assay mixture contained the components described in [7] with minor modifications. The reaction was initiated by adding 1 mM of L-homoserine to the mixture. Under these conditions, the optimum pH for the reaction was found to be 7.5 (Hepes buffer 0.1 M) and the optimum temperature was 30°C. Protein concentrations were determined by the method of Lowry [10].

2.6.2. Assay of AK

Aspartate kinase activity was measured by the same assay and at the same pH and temperature conditions used for HSK, except that 10 mM ATP was used and that L-homoserine was replaced by 10 mM L-aspartate. The ASK activity is optimal between pH 5 and 9 [11].

2.6.3. Inhibition of activity by different amino acids

The initial velocity of each enzyme was measured adding different amino acids to reaction mixtures prepared as before except that 0.25 mM of L-homoserine and 5 mM of ATP was used for the HSK assay, while 4 mM of L-aspartate and 10 mM of ATP was used for the ASK assay. The resulting velocity was compared to that obtained in similar conditions but in the absence of the corresponding amino acid, considered as 100%.

3. RESULTS AND DISCUSSION

3.1. *Specificity of the enzymatic assay*

The P45 fraction obtained as described in Section 2 during the enzyme purification from a wild type strain, contained the HSK (259 mU/mg protein) and the ASK (162 mU/mg protein) activities. These activities were in both cases 90–95% dependent on the addition of the respective substrate, L-homoserine or L-aspartate.

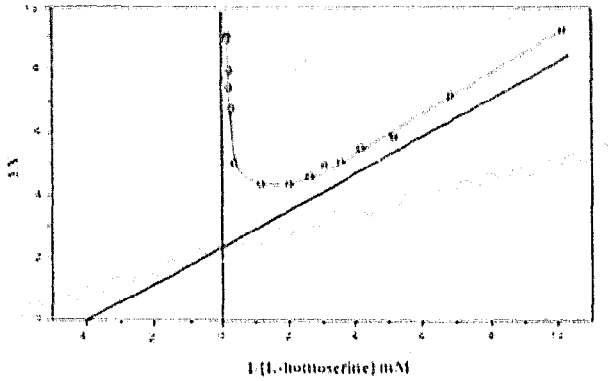

Fig. 1. Plot of the reciprocal of initial velocity (v) of the homoserine kinase reaction versus the reciprocal of the molar concentration of L-homoserine at fixed concentration of ATP (5 mM). v is expressed as $-\Delta OD_{340}$/min. Homoserine kinase was purified from the wild-type strain X2180-1A.

Moreover, a P45 fraction obtained from strain F15 (Thr1$^-$Hom3$^+$) showed a HSK and an AK specific activity of 0 and 225 mU/mg protein, respectively. Conversely, a P45 fraction obtained from strain D160-2C (Thr1$^+$Hom3$^-$) showed a HSK and an ASK specific activity of 108 and 6 mU/mg protein, respectively. These results demonstrate that the described purification protocol and enzymatic assays are sufficiently specific and efficient as to be a useful tool in the quick characterization of both enzymatic activities from a yeast strain.

3.2. *General properties of HSK and ASK*

The partially purified HSK was used to carry out initial velocity studies on the behaviour of the physiological substrates, ATP and L-homoserine. Whereas ATP displayed normal Michaelis-Menten saturation kinetics (K_m = 0.6 mM), L-homoserine shows a hyperbolic saturation kinetics up to a concentration of 0.5 mM, beyond which partial inhibition activity was observed. A double-reciprocal plot of the initial velocity versus the molar concentration of L-homoserine (Fig. 1) allows a calculation of an apparent K_m and K_i for L-homoserine of 0.25 mM and 2 mM, respectively [12]. These parameters are very similar to those reported for the *E. coli* enzyme. Inhibition by saturating concentrations of homoserine could play a physiological role in the regulation of the homoserine kinase if, as described by Seibold et al. [13], the internal concentration of homoserine in yeast cells grown on ammonium sulphate approaches 3 mM.

Similar studies carried out in order to determine the kinetic parameters of the AK showed that both substrates, ATP and L-aspartate, displayed normal Michaelis-Menten saturation kinetics, with a K_m = 3.6 mM and 4 mM, respectively.

3.3. *Inhibition by threonine and other amino acids*

As shown in Table I, of all natural amino acids

Table I

Inhibition of the homoserine kinase (HSK) and the aspartate kinase (ASK) activities from strains X2180-1A (wild type) and F15 (Thr1⁻ Hom3⁻) by different amino acids related with the threonine biosynthetic pathway

Amino acid	Concentration (mM)	Inhibition (%)		
		HSK	ASK	
		X2180-1A *THR1 HOM3*	X2180-1A *THR1 HOM3*	F15 *thr1 HOM3*
L-Threonine	20	62	94	70
	2	34	nd	nd
Hydroxynorvaline[a]	50	11	93	83
	5	nd	29	nd
L-Methionine	20	13	15	0
L-Isoleucine	20	17	25	0
L-Valine	20	24	5	0
	2	0	nd	nd
L-Serine	20	17	1	0
	2	0	nd	nd
L-Homoserine	20	27	2	0
L-Lysine	20	nd	nd	0

[a] Mixture of the 4 diastereoisomers

nd, not determined

tested, only threonine inhibits effectively both the ASK and the HSK activities. More extensive data on this inhibition, presented in Fig. 2, indicate that the L-threonine effect on HSK seems to be only partial, leaving, even at the higher concentrations tested, around 25% activity. The L-threonine concentration required for half-maximal inhibition was about 10 mM. The variation of HSK activity, as a function of L-homoserine concentration in the presence of different quantities of L-threonine, clearly shows that this inhibition is competitive (Fig. 3). From these data, a K_i of 8.7 mM can be estimated [12]. Such an inhibition is similar to that found in the *E. coli* HSK (K_i = 10 mM) [8,9]. However, while the *E. coli* HSK is also inhibited by several other amino acids and threonine analogs (like hydroxynorvaline), the *S. cerevisiae* HSK seems to be rather insensitive to them. These results argue against the HSK playing an important role in the regulation of the threonine biosynthesis at least at the enzymatic activity level.

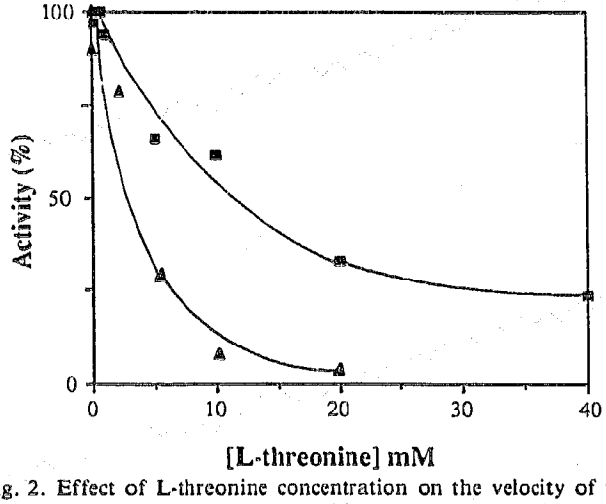

Fig. 2. Effect of L-threonine concentration on the velocity of the homoserine kinase (■) and aspartate kinase (▲) activities of the wild-type strain X2180-1A.

Table I also shows that the yeast ASK activity is not affected by L-methionine but it is strongly inhibited by L-threonine, being 3 mM the concentration required for half-maximal inhibition (see Fig. 2). These results agree with those reported before by de Robichon-Szulmajster and Corrivaux [2] who also mention that this inhibition is allosteric. However, the inhibition of 30–40% exerted by 10 mM L-homoserine, also reported by these authors, could not be found, neither in a Thr1⁻, nor in a wild type strain (Table I), in which the added homoserine could be used by the HSK in the assay and hide its inhibitory action over the ASK. Taking also into account that the results obtained with any method used admits a standard deviation of around 15%, we consider that this inhibition is probably not significant.

Of the other amino acids tested, only hydroxynorvaline, a structural threonine analog, inhibits the ASK activity (Table I).

De Robichon-Szulmajster et al. propose the existence

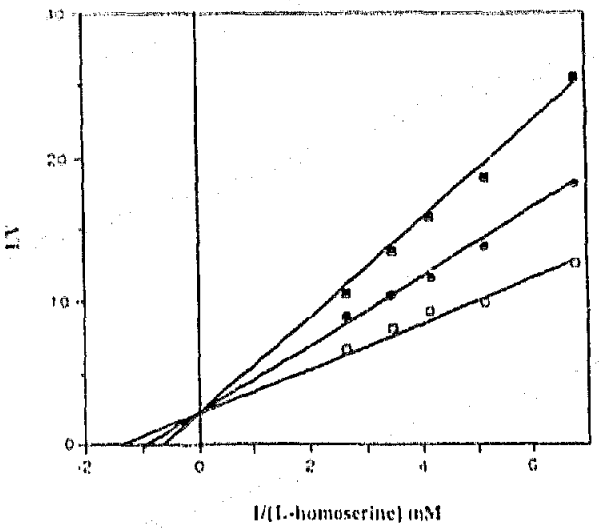

Fig. 3. Double-reciprocal plot of the inhibition of homoserine kinase activity of the wild-type strain X2180-1A by L-threonine at different concentrations of L-homoserine. (□) No L-threonine; (●) 5 mM L-threonine; (■) 10 mM L-threonine. v is expressed as $-\Delta OD_{340}$/min.

in yeast of two ASK, A and B. ASK A, synthesized in all culture conditions, would be responsible for at least 80% of the ASK activity, and only sensitive to inhibition by threonine and homoserine. The synthesis of ASK B would be induced by high concentrations of L-methionine (20 mM) in the culture medium, and its activity inhibited by L-methionine (around 20% at 10 mM) and by L-lysine (around 25% at 20 mM) [2,14]. A *S. cerevisiae* cell growing on ammonium sulphate, accumulates approximately 5 mM of threonine and 0.4 mM of methionine [1]. Thus, in normal circumstances, ASK B would be practically absent and the transformation of aspartate into aspartylphosphate would be in fact carried out by the ASK A. In this case, the possible inhibition caused by methionine or lysine of the ASK B activity would be completely irrelevant for the control of the pathway. This view is also supported by the existence of *hom3* mutants, which lack ASK activity (see above), presumably because they are defective in the main ASK [15]. These mutants are unable to grow in a medium lacking threonine even in the presence of methionine which would induce the synthesis of the secondary ASK. As stated by Stadtman et al. [14], the results could be also consistent with the view that yeast contains a single ASK showing different sensitivities depending on the culture medium. Another interpretation would be that a single ASK exists and that the observed inhibition by methionine and lysine might correspond to another enzyme, present in the reaction mixture but independent from the real ASK.

The strong inhibition exerted by threonine on the ASK activity argues for this enzyme and for threonine, the final product, as the main agents in the regulation of this biosynthetic pathway.

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