THE ACTION OF O-METHYL-THREONINE AND THIAISOLEUCINE ON THREONINE DEAMINASE PURIFIED FROM *ESCHERICHIA COLI* K-12

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Received 12 July 1972

1. Introduction

O-methyl-threonine and thiaisoleucine (2-amino-3-methyl-thiobutyrate) are two analogues of isoleucine which inhibit the growth of *Escherichia coli:* O-methyl-threonine inhibition is reversed by isoleucine, valine and leucine [1] while thiaisoleucine inhibition is reversed by isoleucine alone [2]. It has been suggested that these two substances might inhibit the growth of *E. coli* in different ways: they might interfere with the active transport of isoleucine into the cell, compete with isoleucine for incorporation into proteins, cause a "false repression" of "false feedback inhibition" [1, 2]. These two substances also inhibit the activity of the enzyme threonine deaminase: inhibition of threonine deaminase by O-methyl-threonine in crude extracts of *E. coli* 15 has been reported by Smulson et al. [1] while inhibition of threonine deaminase by thiaisoleucine in crude extracts of *E. coli* K-12 has been reported by Szentirmai and Umbarger [2]. This inhibition might be a consequence of the similarity in structure between O-methyl-threonine thiaisoleucine and isoleucine (see table 1), since it has been reported [3, 4] that the latter substance is an allosteric inhibitor of threonine deaminase. It is not possible, however, to conclude from the available data if these two analogues interact with threonine deaminase at the allosteric site (and thus behave as isoleucine analogues of "false feedback" inhibitors) or at the catalytic site (in which case they should be considered threonine analogues).

Since "false feedback" inhibitors are useful analogues to be used in the search for regulatory mutants and, since such inhibitors are not known in the case of isoleucine biosynthesis, we decided to test the effect of these two analogues on the activity of threonine deaminase (L-threonine-hydrolase (deaminating) EC 4.2.1.16) purified from *E. coli* K-12. This allosteric [5] enzyme is the first enzyme in isoleucine biosynthesis (see table 1); its allosteric inhibitor is isoleucine while valine is an allosteric activator. In this report we illustrate experiments on the mechanism of inhibition of threonine deaminase by O-methyl-threonine and thiaisoleucine.

2. Materials and methods

2.1. *Reagents*

Amino acids used were all L-form of the highest purity available. Uridine was purchased from Sigma. Thiamine hydrochloride, dithiothreitol (Cleland's reagent) and pyridoxal phosphate were purchased from Calbiochem. NADH and lactate dehydrogenase

Table 1
Isoleucine biosynthetic pathway and the structure of O-methyl-threonine and thiaisoleucine.

COOH CH–NH$_2$ CH–OH CH$_3$	Threonine deaminase →	COOH C = O CH$_2$ CH$_3$
L-Threonine		α-Ketobutyrate

→ → → →

COOH CH–NH$_2$ CH–CH$_2$–CH$_3$ CH$_3$
L-Isoleucine

COOH CH–NH$_2$ CH–O–CH$_3$ CH$_3$	COOH CH–NH$_2$ CH–S–CH$_3$ CH$_3$
O-Methyl-threonine	Thiaisoleucine

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were obtained from Boehringer Mannheim GmbH, DEAE-cellulose and hydroxylapatite were products of Bio Rad. Thiaisoleucine hydrochloride (2-amino-3-methyl-thiobutyrate) and O-methyl-DL-threonine were obtained from Reef Laboratories, Santa Paula, Calif. It is not known if thiaisoleucine is a racemic form [2]. All other chemicals were reagent grade, and quartz-redistilled water was used for all solutions.

2.2. *Bacterial strain and culture conditions*

Threonine deaminase was purified as described in the next section from a derivative of *E. coli* K-12, strain MI164, a HfrH bearing the following chromosomal markers: *pyrA53, thi-1,* $(\lambda)^-$, *ileS1, ilv0.* The preparation of this strain has been described by Iaccarino and Berg [6]: it is a Thr^+ transductant (isolate no. 18) of strain AW206 transduced with a P1 phage grown on strain MI1 *(ileS1, thr$^+$).* Strain MI164 still contains the *ilv0* mutation of AW206 but has now also an altered isoleucyl-tRNA synthetase activity due to the presence of the *ileS1* mutation; as a consequence of the presence of these two mutations threonine deaminase is more derepressed than in either AW206 or MI1.

Strain MI164 was grown in the minimal medium described by Vogel and Bonner [7] supplemented with 100 mg/l of L-arginine, 100 mg/l of L-isoleucine, 50 mg/l of uridine, 10 mg/l of thiamine hydrochloride and 50 mg/l of L-leucine. Leucine was added because, as described by Guardiola and Iaccarino [8], we usually observe a 2- to 3-fold derepression of threonine deaminase upon addition of leucine to the minimal medium.

Typical specific activities (see sect. 2.3) of extracts were 0.046 for wild type, 0.480 or 1.09 for strain MI164 grown, respectively, in minimal medium with no leucine added or in minimal medium supplemented with leucine. Our initial extracts, therefore, are about 24-fold derepressed as compared to wild type.

2.3. *Enzyme assay*

Threonine deamination in crude extracts was assayed by measuring the rate of formation of α-ketobutyrate as described by Iaccarino and Berg [6]. Rate studies with the partially purified enzyme were performed by the use of the coupled assay of Maeba and Sanwal [9] in a final volume of 0.8 ml. In this assay mixture, containing an excess lactate dehydrogenase, the α-ketobutyrate produced in the threonine deaminase reaction oxidizes NADH and the rate of oxidation, measured spectrophotometrically at 340 nm, is proportional to the amount of threonine deaminase added. The reaction was started by addition of enzyme.

A unit of enzyme activity is defined as the amount of enzyme forming at 22° 1 μmole of α-ketobutyrate per minute. Specific activities are units per mg of protein. Proteins were determined by the method of Groves [10] with crystalline bovine serum albumin as a standard.

Table 2
Purification of threonine deaminase from *E. coli* K-12.

	Volume (ml)	Total activity (units)	Total proteins (mg)	Specific activity (units/mg)	Yield (%)
Extract	56	3320	3046	1.09	100
DEAE-cellulose	260	1370	135.2	10.0	41
Hydroxyl-apatite	12	686	11.3	60.8	21

2.4. *Enzyme purification*

Threonine deaminase was purified approx. 60-fold from crude extracts, representing 1300-fold purification over the activity of wild type extracts. The purification procedure is identical to that described by Burns and Zarlengo [11] for the enzyme from *Salmonella*

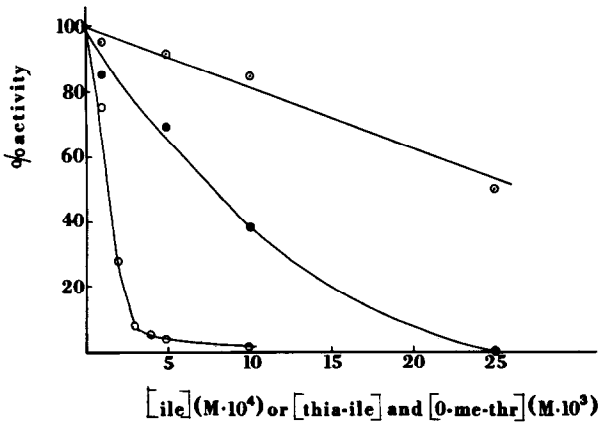

Fig. 1. Inhibition of threonine deaminase activity by isoleucine (ile: ○—○—○), O-methyl-threonine (O-me-thr: ⊙—⊙—⊙), thiaisoleucine (thia-ile: ●—●—●). Each assay was performed with 0.94 μg of purified enzyme.

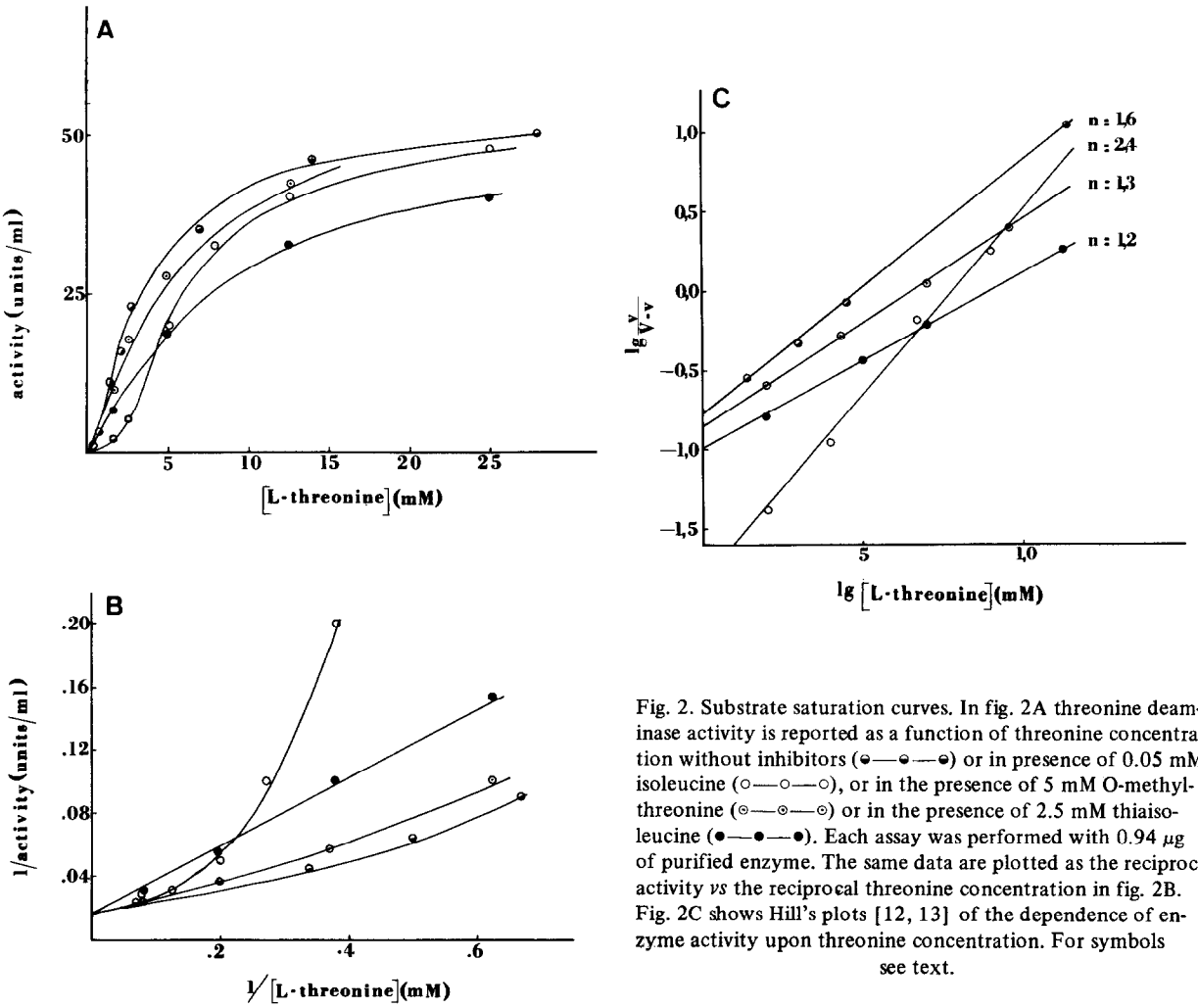

Fig. 2. Substrate saturation curves. In fig. 2A threonine deaminase activity is reported as a function of threonine concentration without inhibitors (●—●—●) or in presence of 0.05 mM isoleucine (○—○—○), or in the presence of 5 mM O-methyl-threonine (⊙—⊙—⊙) or in the presence of 2.5 mM thiaisoleucine (●—●—●). Each assay was performed with 0.94 μg of purified enzyme. The same data are plotted as the reciprocal activity *vs* the reciprocal threonine concentration in fig. 2B. Fig. 2C shows Hill's plots [12, 13] of the dependence of enzyme activity upon threonine concentration. For symbols see text.

typhymurium and includes column chromatography on DEAE-cellulose and hydroxylapatite. The enzyme fractions from the hydroxylapatite column were pooled and dialyzed against 0.05 M potassium phosphate pH 7.4, containing 8×10^{-4} M L-isoleucine, 5×10^{-4} M EDTA and 5×10^{-4} M Cleland's reagent and stored at −20°.

Table 2 shows results of a typical purification. The purified enzyme preparation shows a negligible oxidation of NADH when no threonine is present (less than 0.6%). Although this is the best preparation to our knowledge described in the literature for threonine deaminase from *E. coli*, it is probably far from being pure.

3. Results and discussion

The inhibition of threonine deaminase activity by isoleucine, O-methyl-threonine and thiaisoleucine is shown in fig. 1; concentrations at which 50% inhibition is achieved are 0.14 mM for isoleucine, 25 mM for O-methyl-threonine, and 7.5 mM for thiaisoleucine; K_i's were not calculated because Lineweaver and Burk's plots were not linear (see fig. 2B).

The inhibitory effect of isoleucine, O-methyl-threonine and thiaisoleucine was also studied as a function of substrate concentration and the results are shown in fig. 2A. It appears that isoleucine increases the sigmoid character of the saturation curve of enzyme with re-

spect to the substrate; O-methyl-threonine and thiaisoleucine do not show this effect and therefore they should not be considered allosteric inhibitors. In fig. 2B the same data are plotted as reciprocal enzyme activity *vs* reciprocal substrate concentration. As expected, the experimental points obtained with no inhibitors do not fit a straight line since the enzyme preparation used contains isoleucine (final conc. in these assays: 1×10^{-6}M, see Materials and methods). Addition of isoleucine increases the deviation from linearity in agreement with previously published data [3] while O-methyl-threonine and thiaisoleucine do not show this effect.

In fig. 2C data of the substrate saturation curves are plotted as $\log \frac{v}{V-v}$ against log [threonine] in order to determine the apparent order of the reaction with respect to the substrate, either in the absence of effectors, or in the presence of the allosteric inhibitor isoleucine, or in the presence of one of the two other inhibitors O-methyl-threonine and thiaisoleucine, according to the following equation

$$\log \frac{v}{V-v} = \log K + n \log [\text{threonine}]$$

(where V = activity (v) in the presence of saturating substrate) [12, 13]. The order of the reaction n for the substrate is equal to 1.6 when no inhibitors are present; in the presence of isoleucine it increases to a value of 2.4, while it does not increase in the presence of O-methyl-threonine (n = 1.3) or thiaisoleucine (n = 1.2). Thus these values of n support the conclusion that O-methyl-threonine and thiaisoleucine are competitive inhibitors of threonine deaminase. If this conclusion is correct it should be possible to confirm it by the use of the other allosteric effector, valine: it has been reported, indeed, that isoleucine inhibition of threonine deaminase is counteracted by the other allosteric effector valine [3]; if O-methyl-threonine and thiaisoleucine are isosteric inhibitors their inhibition should not be counteracted by valine. Fig. 3 shows that valine is able to remove isoleucine inhibition, but not the inhibition caused by O-methyl-threonine and thiaisoleucine.

O-methyl-threonine and thiaisoleucine appear to interact at the active site of threonine deaminase. Unpublished evidence suggesting the same conclusion for thiaisoleucine has been reached by Dr. H.E. Umbarger (personal communication). Evidence confirming this

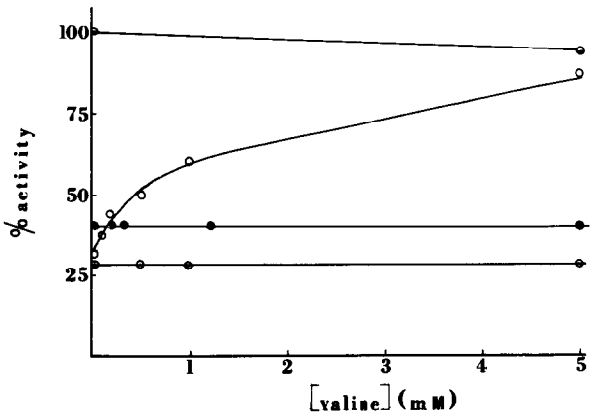

Fig. 3. Effect of valine on the inhibition of threonine deaminase activity exerted by isoleucine, O-methyl-threonine of thiaisoleucine. (●—●—●): control (no inhibitor added); (○—○—○): 0.2 mM isoleucine; (◎—◎—◎): 40 mM O-methyl-threonine; (●—●—●): 10 mM thiaisoleucine. Each assay was performed with 0.94 μg of purified enzyme.

conclusion for O-methyl-threonine has been reported by Smulson et al. [1] who showed that when threonine deaminase in extracts of *E. coli* 15 is treated with Hg^{2+} it becomes insensitive to isoleucine but it is still inhibited by O-methyl-threonine. It is possible to conclude, therefore, that O-methyl-threonine and thiaisoleucine behave as threonine analogues in the case of this enzyme. On the other hand these two substances behave as isoleucine analogues in other systems. It has been reported, in fact, that O-methyl-threonine and thiaisoleucine behave as substrates for isoleucyl-tRNA synthetase either extracted from *E. coli* 15 [1] or *E. coli* K-12 [2] or purified from *E. coli* K-12 [14]; they also inhibit the active transport of valine into *E. coli* K-12 (Guardiola, Klopotowski and Iaccarino, unpublished experiments) while thiaisoleucine inhibits the transamination of isoleucine [2]. It is apparent therefore that the definition of analogue of a certain natural product is related to the enzyme or system tested.

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