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The Feedback Control Mechanism of Biosynthetic L-Threonine Deaminase by L-Isoleucine

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"Man does not realize how that which varies is a unity. There is a harmony of opposite tensions as there is one of bow and lyre."

Heraclitus the Obscure.

I. INTRODUCTION

As has been extensively discussed in other reports of this Symposium, the biosynthesis of metabolites in microorganisms is controlled by negative feedback mechanisms. In the case of feedback inhibition the end-product competitively inhibits the activity of the first specific enzyme of a chain—and consequently blocks the enzymatic processes leading to its own synthesis. Before the discovery of such regulatory enzymes, only analogues of substrates were known as competitive inhibitors. Thus the problem arose: how can a compound with a structure very dissimilar from that of its substrate act as a competitive inhibitor of an enzyme?

Our investigation concerns the first enzyme specifically involved by Escherichia coli K12 in the biosynthesis of isoleucine: L-threonine deaminase, which converts L-threonine into α-ketobutyric acid. As observed by Umbarger (1956; Umbarger and Brown, 1957b), the activity of this biosynthetic L-threonine deaminase is competitively inhibited by L-isoleucine, thus providing a negative feedback mechanism controlling the biosynthesis of this amino acid.

Just as for other enzymatic systems involved in metabolite biosynthesis, the end-product competitively inhibits the activity of the first specific enzyme of a chain—and consequently blocks the enzymatic processes leading to its own synthesis. Before the discovery of such regulatory enzymes, only analogues of substrates were known as competitive inhibitors. Thus the problem arose: how can a compound with a structure very dissimilar from that of its substrate act as a competitive inhibitor of an enzyme?

Our investigation concerns the first enzyme specifically involved by Escherichia coli K12 in the biosynthesis of isoleucine: L-threonine deaminase, which converts L-threonine into α-ketobutyric acid. As observed by Umbarger (1956; Umbarger and Brown, 1957b), the activity of this biosynthetic L-threonine deaminase is competitively inhibited by L-isoleucine, thus providing a negative feedback mechanism controlling the biosynthesis of this amino acid.

II. EXPERIMENTAL

If L-isoleucine is a competitive inhibitor of L-threonine deaminase, an excess of L-isoleucine should exclude L-threonine from the active site for deamination, and more conclusively, L-threonine should displace L-isoleucine from its complex with the enzyme. Figure 1 shows that for a given concentration of L-threonine, an excess of L-isoleucine removes the deaminase activity completely and that when the substrate concentration is doubled, approximately twice as much inhibitor is required to obtain 50% inhibition. Furthermore, if the velocity of deamination is measured at increasing concentrations of L-threonine in the presence of the same level of L-isoleucine, the curves drawn may be extrapolated to the same Vmax, value (Fig. 2). Thus it can be concluded that the inhibitory effect of L-isoleucine is competitive.

However, the kinetics of L-threonine deamination both in the presence and absence of L-isoleucine are somewhat complex:

a. As already described by Umbarger (Umbarger and Brown, 1957b), the variation of the initial rate of L-threonine deamination as a function of substrate concentration does not follow simple Michaelis-Menten kinetics, as shown clearly by the use of the double reciprocal plot. The plotted curve diverges considerably from a straight line and is approximated by a parabola. A similar graph is also obtained when the rate of L-threonine deamination is measured in the presence of low concentrations of L-isoleucine. However, when the concentration of L-isoleucine in the incubation medium is high, a straight line fits the data plotted by the method of Lineweaver and Burk (Fig. 2).

b. If the velocity of deamination measured in the presence of increasing concentrations of L-isoleucine is plotted against the concentration of L-isoleucine, the curve obtained (Fig. 1) has two parts, expressing apparently different kinetic properties of the system. This is more evident when the reciprocal of the activity is plotted against the concentration of L-isoleucine: the part of the curve for low concentrations of L-isoleucine is a straight line while that for high levels of L-isoleucine becomes a parabola. These complex data indicate an anomalous binding of both L-isoleucine and L-threonine to the enzyme which appears to
be compatible within a certain range with a bimolecular reaction with respect to both substrate and inhibitor.

In summary, while data show that L-isoleucine is a competitive inhibitor of L-threonine deaminase, the kinetic behavior of this enzyme deviates from normal. One is tempted to speculate that this peculiar behavior is correlated with the steric properties involved in feedback control. The action of L-threonine and L-isoleucine on the same enzymatic system, in spite of their molecular dissimilarity, leads to the hypothesis that distinct binding groups specific for each exist on the surface of the deaminase enzyme. Therefore, we attempted to obtain a preparation which would retain its action on L-threonine but would be insensitive to L-isoleucine.

In a first attempt to obtain modified L-threonine deaminase, the system was purified by fractionation with ammonium sulfate, heat-treatment in the presence of high concentrations of L-isoleucine, and phosphate gel adsorption. The final preparation had a specific activity 200 times higher than the crude extract of the wild type. A better purification scheme has not yet been achieved.

The kinetic properties of the enzyme in this fraction proved to be significantly different from that of the native enzyme.

1. Using the purified L-threonine deaminase, the reaction kinetics now obey classical Michaelis-Menten kinetics: the data can be plotted as a straight line according to the Lineweaver and Burk method, allowing the determination of an approximate $K_m$ value of $5 \times 10^{-4}$ M for L-threonine.

2. The inhibitory effect of L-isoleucine on the purified deaminase also appears to be modified: when the deaminase activity for a given concentration of L-threonine is plotted against increasing L-isoleucine concentrations, a curve without an angular point can be drawn (Fig. 3), and an excess of L-isoleucine does not completely remove deaminase activity. (The possibility that a minor component, with properties different from the native biosynthetic L-threonine deaminase, might have been concentrated during the purification appears unlikely since the purification resulted in less than 60% loss of the activity of the crude extract.) Thus the biosynthetic L-threonine deaminase was apparently modified by purification in its sensitivity to L-isoleucine, although it is possible

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**Figure 1.** Effect of increasing concentrations of L-isoleucine on L-threonine deaminase activity from crude extract of derepressed mutant. The reaction mixture contained: M/50 L-threonine (curve 2) or M/25 L-threonine (curve 1), 0.1 μg pyridoxal phosphate, $10^{-4}$ M magnesium titriplex, 0.2 M sodium phosphate buffered at pH 8.0, 45 μg protein crude extract of *Escherichia coli* K 12 derepressed mutant for the biosynthetic L-threonine deaminase. Total volume: 1.0 ml. The experiments were carried out at 27°C. The keto acid production was determined by the method of Friedemann and Haugen.

**Figure 2.** Rate dependence of α-ketobutyrate formation on L-threonine concentration and on L-isoleucine. Crude extract of derepressed mutant. Double reciprocal plot. Assay as in Fig. 1, except for substrate and inhibitor concentrations.
that the purified fraction was not homogeneous in this respect.

In an effort to obtain an enzyme totally insensitive to L-isoleucine inhibition, various treatments were attempted.

When the purified dialyzed enzyme is heated in the presence of molar sodium phosphate pH 8.2, the activity is preserved while the sensitivity to L-isoleucine quickly disappears (Fig. 4). (This fraction no longer inhibited by L-isoleucine will be called "desensitized L-threonine deaminase").

However, if the heating is done in the presence of L-isoleucine or even L-threonine, the sensitivity to L-isoleucine is largely preserved.

A protective effect against desensitization also occurs if magnesium titriplex, a metal complexing agent, is added to the incubation medium, suggesting that a heavy metal is implicated in this process of desensitization.

The effect of parachloromercuribenzoate (PCMB), a reagent specific for blocking sulfhydryl groups, was therefore tested. The crude or purified enzyme fractions were incubated in the presence of PCMB; part of the activity was lost during this treatment, but the remaining deaminase activity was not detectably inhibited by L-isoleucine (Tables 1 and 2).

If the PCMB treatment is performed in the presence of L-isoleucine, a certain loss of enzymatic activity is observed; however, no desensitization occurs (Table 2).

These data indicate that partially different groups—particulary SH groups—are involved in the binding of L-threonine and L-isoleucine, respectively. However, it remains true that L-isoleucine competitively inhibits L-threonine deaminase activity. This com-

Table 1. Effect of PCMB on Sensitivity to L-Isoleucine Inhibition of L-Threonine Deaminase from Crude Extract

<table>
<thead>
<tr>
<th>PCMB concentration</th>
<th>μM α-Ketobutyric acid produced in the presence of</th>
<th>% inhibition by L-Isoleucine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-threonine</td>
<td>L-threonine + L-Isoleucine</td>
</tr>
<tr>
<td>0</td>
<td>130.0</td>
<td>00.0</td>
</tr>
<tr>
<td>10^-9 M</td>
<td>43.5</td>
<td>29.0</td>
</tr>
<tr>
<td>7.5 × 10^-9 M</td>
<td>22.2</td>
<td>23.0</td>
</tr>
</tbody>
</table>

Figure 3. Effect of increasing concentrations of L-isoleucine on L-threonine deaminase activity from a purified fraction. Assay as in Fig. 1: M/50 L-threonine, enzyme concentration was 20 μg protein/ml.

Figure 4. Effect of temperature on sensitivity to L-isoleucine inhibition of L-threonine deaminase from a purified fraction. The dialyzed enzyme at a concentration of 0.25 mg Protein/ml was equilibrated at 55°C for the indicated time in the presence of molar sodium phosphate buffered at pH 8.2, after which the enzymatic activity against M/50 L-threonine was measured in the presence of 10^-3 M L-isoleucine (1) and in the absence of inhibitor (2). The enzyme concentration was 25 μg protein/ml.
The purified fraction carefully dialyzed at a concentration of 0.30 mg Protein/ml was treated at 0°C for 30 min by 2.5 × 10⁻⁵M PCMB in the presence of 10⁻³M L-isoleucine or in the absence of inhibitor. The incubation medium contained molar sodium phosphate buffered at pH = 8.0. The treated fraction was immediately diluted 10 times with the assay medium. Assay in the presence of M/50 L-threonine.

<table>
<thead>
<tr>
<th></th>
<th>mM of Ketobutyrlic acid produced in the presence of</th>
<th>% inhibition by L-Isoleucine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O Isoleucine</td>
<td>10⁻³ M L-Isoleucine</td>
</tr>
<tr>
<td>No PCMB</td>
<td>140</td>
<td>50</td>
</tr>
<tr>
<td>2.5 × 10⁻⁵M PCMB + 10⁻³M L-Isoleucine</td>
<td>72.5</td>
<td>27.5</td>
</tr>
<tr>
<td>2.5 × 10⁻³M PCMB</td>
<td>37.5</td>
<td>32.0</td>
</tr>
<tr>
<td>No L-Isoleucine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Competitive inhibition implies that important interactions occur between the binding locations of L-isoleucine and L-threonine. Other data also illustrate the existence of these interactions:

1. As previously mentioned, heat treatment in the presence of L-threonine no longer renders the purified enzyme insensitive to L-isoleucine (Table 3). Furthermore, it can easily be demonstrated that L-isoleucine strongly protects native L-threonine deaminase against thermal inactivation (Fig. 5).

2. As already shown, the L-threonine deaminase activity of the native enzyme does not follow Michaelis-Menten kinetics. In contrast, the thermally desensitized enzyme exhibits classical kinetics (Fig. 6). With the native enzyme, a concentration of 2.7 × 10⁻⁵ M L-threonine is sufficient to reach V_max/2 while a concentration of 1.4 × 10⁻³ M L-threonine is necessary with the desensitized fraction.

Thus, when certain binding groups apparently specific for L-isoleucine are altered, the active center for L-threonine deamination is simultaneously modified.

Similar experimental data have been independently obtained by Pardee (personal communication) working with aspartate transcarbamylase.

### Table 3. Effect of L-Threonine and Mg Titriplex on Thermal Desensitization of L-Threonine Deaminase from a Purified Fraction

Same conditions as in Fig. 4. Heat treatment for 10 min. The slight discrepancies with the results of Fig. 4 may be attributed to the quality of the phosphate buffer.

<table>
<thead>
<tr>
<th>Heat treatment in the presence of</th>
<th>mM of Ketobutyrlic acid produced in the presence of</th>
<th>% inhibition by L-Isoleucine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O Isoleucine</td>
<td>10⁻³ M L-Isoleucine</td>
</tr>
<tr>
<td>M/200 L-threonine</td>
<td>54.6</td>
<td>16.0</td>
</tr>
<tr>
<td>10⁻³M Mg Titriplex</td>
<td>55.5</td>
<td>12.0</td>
</tr>
<tr>
<td>None</td>
<td>28.0</td>
<td>19.6</td>
</tr>
<tr>
<td>No heat treatment</td>
<td>62.5</td>
<td>12.5</td>
</tr>
</tbody>
</table>

### III. CONCLUSIONS

The main results may be summarized as follows:

1. Inhibition by L-isoleucine of L-threonine deaminase is competitive although there is dissimilarity of structure between L-threonine and L-isoleucine.

2. When the native enzyme is submitted to certain treatments, it loses sensitivity to L-isoleucine while retaining L-threonine deaminase activity.

3. The native enzyme exhibits complex kinetics, which suggests bimolecular reaction with respect to both substrate and inhibitor. The desensitized enzyme...
In order to account for the latter result, it seems inevitable to assume the existence of two distinct sites which we would respectively designate as activity site (A) and inhibition site (I) and to further assume that the properties of the activity site are influenced by the compound bound at the inhibition site. A model of this type which would be compatible with the results is shown by Fig. 7, model 2.

This model involves the following assumptions regarding the properties and the interaction of the two sites:

1. The enzyme is active when L-threonine is bound at the activity site.
2. The affinity for L-threonine of the activity site is increased when L-threonine also occupies the inhibition site.
3. The affinity of the activity site is decreased when the inhibition site is occupied by L-isoleucine.
4. L-isoleucine presents low affinity for the activity site.

Given these assumptions it would be clear that, at relatively low concentrations of L-threonine, the kinetic behavior will be normal (α). At high concentrations of L-threonine the apparent affinity for substrate increases, giving rise to the apparent “bimolecular effect” (β).

At relatively low concentrations of L-isoleucine, L-threonine can still be bound by the enzyme (γ). When the L-isoleucine concentration increases, L-threonine is altogether excluded, hence the “bimolecular effect” of L-isoleucine (δ).

This model is complex, and the properties assumed for the two sites are admittedly ad hoc. However, it does not seem possible to account for the facts with a simple set of assumptions.

In any case, the most distinctive assumption in this model is that two molecules of L-threonine or two of L-isoleucine, or one of L-isoleucine and one of L-threonine, may be bound simultaneously to the en-
zyme. One may hope to apply this assumption to the experimental test.

Whatever the interpretation of this effect may be, it is worth noting that as a result of the "bimolecular" kinetics of inhibition the effect of metabolite becomes significant above a threshold value. In other words, the intracellular metabolic pool should become effective in the feedback system only when the concentration rises above a critical level. Many physiological phenomena would thus be dependent on the kinetics of this important control mechanism.

REFERENCES
PARDEE, A. B., personal communication.


DISCUSSION
DAVIS: The properties of threonine dehydrase suggest an analogy to hemoglobin. Pauling has analyzed the mechanism by which each molecule of O₂ taken up by this compound affects its affinity for the next molecule of O₂. The 4 interdependent affinity constants of hemoglobin thus give rise to its characteristic sigmoid O₂ dissociation curve, so elegantly adapted to unloading a large fraction of its bound oxygen in the tissues without requiring an excessive drop in pO₂. Dr. Changeux’s work suggests a similar mechanism, with two sites, to account for the sensitivity of a control enzyme in bacteria to the square of the concentration of its endproduct. The evolution of such similar mechanisms in a control enzyme and in hemoglobin should not be surprising, since the function of feedback inhibition, in stabilizing the concentration of a metabolite within a narrow range, has a certain resemblance to the function of hemoglobin.