Acyl Derivatives of Homoserine as Substrates for Homocysteine Synthesis in Neurospora crassa, Yeast, and Escherichia coli*

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SUMMARY

"Activated" forms of L-homoserine have been shown to be more readily utilized as substrates than homoserine for the homocysteine synthetases (enzymes which catalyze the formation of homocysteine from homoserine and sulfide in the presence of pyridoxal phosphate) of Neurospora crassa, yeast, and Escherichia coli KB. This substrate preference is shown by the increased specific activities of the enzyme reactions. Specificity for the type of acylated derivative of homoserine is apparent. Enzymes from the fungi preferentially utilize O-acetyl-L-homoserine, and the enzyme from the bacterium requires O-succinyl-L-homoserine.

Methionineless mutants of Neurospora contain homocysteine synthetases with specific activities nearly equivalent to those of the wild type when the substrate is O-acetylhomoserine.

End product feedback inhibition by L-methionine on the homocysteine synthetases, when the substrates used were acyl derivatives of homoserine, is described for the enzymes from Neurospora and yeast, but was not demonstrated with the enzyme from E. coli.

O-Acetyl-L-serine is a required substrate for cysteine synthesis by an enzyme from E. coli KB. Some preference for O-acetylsersine was exhibited by the enzyme from yeast; however, cysteine synthetase from Neurospora showed no marked preference for this compound over serine.

The enzymatic synthesis of homocysteine from L-homoserine and sulfide by Neurospora crassa has been reported (1). An analogous reaction with L-serine and sulfide to form cysteine with the same enzyme preparation was also described. The observation that the specific activity of the enzyme preparation was much higher for the latter reaction than the former suggested that an activated form of homoserine might be a more favorable substrate for homocysteine synthesis. In this report it is shown that O-acetyl-L-homoserine is a more readily utilizable substrate than the unacylated compound for homocysteine synthesis by enzymes in Neurospora and yeast. Furthermore, O-succinyl-L-homoserine can be readily used by an Escherichia coli enzyme for which homoserine is inactive as substrate. Thus, it appears that there is a marked specificity in the requirement for the different derivatives of homoserine by the enzymes from fungi and bacteria.

The biological significance of the activated forms of homoserine is becoming apparent. O-Succinylhomoserine has been shown to be required for cystathionine synthesis in enzymes from E. coli (2, 3) and Salmonella (4). O-Acetylhomoserine is required for cystathionine synthesis via condensation with cysteine by Neurospora (5). The use of either O-succinylhomoserine or O-acetylhomoserine by spinach extracts for cystathionine synthesis has been reported (6). More recently, the synthesis of homocysteine from O-acetylhomoserine and sulfide, and, of cysteine, from O-acetylsersine and sulfide by enzyme extracts of spinach has been demonstrated (7). A brief description of the synthesis of homocysteine from O-acetylhomoserine and sulfide by Neurospora extracts has appeared (8). Our present communication confirms this report. It has also been shown that highly purified cystathionine $\gamma$-synthase from Salmonella typhimurium, which catalyzes the formation of cystathionine from cysteine and O-succinylhomoserine, can catalyze the synthesis of homocysteine directly when H$_2$S replaces cysteine as substrate (9). This reaction was first observed with this organism by Dr. N. Kredich. Since E. coli and S. typhimurium are metabolically very similar organisms, the work we present here corroborates the bacterial synthesis of homocysteine by this route.

The O-acetylated form of L-serine has been reported to be the substrate which accepts sulfide for cysteine synthesis in E. coli and Salmonella (11), although L-serine itself has been shown to function as substrate in this reaction for E. coli (12). In this communication we confirm the preferential use of O-acetylsersine for cysteine synthesis by E. coli and yeast. However,

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the enzyme from Neurospora appears to use serine or its acetylated form equally well.

End product feedback inhibition by methionine on homocysteine synthetase has been demonstrated previously (1). Further work presented here indicates that a similar control exists on the enzymes from Neurospora and yeast when the derivatized forms of homoserine are used as substrates. This phenomenon was not apparent in the case of the enzyme from E. coli.

EXPERIMENTAL PROCEDURE

Materials

L-Homoserine, L-serine, L-methionine, and pyridoxal phosphate were obtained from Calbiochem. Pancreatic deoxyribonuclease I was obtained from Worthington Biochemical Corporation. O-Succinyl-L-homoserine was synthesized according to the method of Flavin and Slaughter (13). The crystallized product had a melting point of 188-189° and \( R_f \) of 0.56 on paper chromatography in 1-butanol-acetic acid-water (70:15:15). L-Homoserine, L-serine, L-methionine, and pyridoxal phosphate were obtained from Calbiochem. Pancreatic deoxyribonuclease (50 /gm per ml) was added, and the preparation was allowed to stand an additional 4 hour. N-Amylamine (1.23 g) was then added, followed by ether (100 ml). On recrystallization from 50% ethanol, the product had a melting point of 163 165°. No contamination by L-serine was observed by paper chromatography. This method was adapted to the synthesis of O-acetyl-L-homoserine as follows. A mixture of 1.75 g of 60.4% HClO₄ and 0.88 g of 98.7% acetic anhydride was diluted to 17.5 ml with 100% glacial acetic acid. L-Homoserine, 1 g, was dissolved in this mixture and quickly cooled to 0°, and 4.2 g of 98.7% acetic anhydride were added. The mixture was allowed to stand 11 hours; 0.85 ml of H₂O was added, and the mixture was allowed to stand an additional 1 hour. N-Amylamine (1.23 g) was then added, followed by ether (100 ml). On recrystallization from 85% ethanol, the product (yield, 1.1 g) had a melting point of 187-189° and \( R_f \) of 0.42 on paper chromatography in 1-butanol-acetic acid-water (90:10:29). L-Homoserine had an \( R_f \) value of 0.25 in this solvent system. The analysis was as follows.

\[
\text{C}_9\text{H}_{15}\text{NO}_4
\]

Calculated: C 45.23, H 6.97, N 8.56

Found: C 44.41, H 6.20, N 6.32

O-Acetyl-L-serine was synthesized according to the method of Greenstein and Winitz (14). On recrystallization from 50% ethanol, the product had a melting point of 163 165°. No contamination by L-serine was observed by paper chromatography. This method was adapted to the synthesis of O-acetyl-L-homoserine as follows. A mixture of 1.75 g of 60.4% HClO₄ and 0.88 g of 98.7% acetic anhydride was diluted to 17.5 ml with 100% glacial acetic acid. L-Homoserine, 1 g, was dissolved in this mixture and quickly cooled to 0°, and 4.2 g of 98.7% acetic anhydride were added. The mixture was allowed to stand 11 hours; 0.85 ml of H₂O was added, and the mixture was allowed to stand an additional 1 hour. N-Amylamine (1.23 g) was then added, followed by ether (100 ml). On recrystallization from 85% ethanol, the product (yield, 1.1 g) had a melting point of 187-189° and \( R_f \) of 0.42 on paper chromatography in 1-butanol-acetic acid-water (90:10:29). L-Homoserine had an \( R_f \) value of 0.25 in this solvent system. The analysis was as follows.

\[
\text{C}_9\text{H}_{15}\text{O}_4\text{N}
\]

Calculated: C 44.72, H 6.83, N 8.69

Found: C 45.23, H 6.97, N 8.56

Methods

Organisms and Culture Methods—Neurospora crassa strains used were wild type Staa4A, methionineless mutants H-98 A (homocysteineless), and 36104a (cystathionineless). Culture methods used were those described previously (1). Escherichia coli KB was maintained on nutrient agar slants. C Medium (15) was used for the cultivation of cells for the enzyme preparation. Yeast, in pressed 1-pound cakes from Anheuser-Busch Company, St. Louis, Missouri, was used.

Enzyme Preparation from Neurospora crassa—The enzyme was prepared and purified through protamine sulfate and ammonium sulfate fractionations as described previously (1). The precipitate from the last ammonium sulfate fractionation was dissolved in 6 \( \times 10^{-4} \) M sodium pyrophosphate buffer, pH 8.0 (1), and applied to a Bio-Gel P 10 column (1.5 x 22 cm) that had been previously washed with 10⁻⁴ M pyridoxal phosphate in the same buffer. The protein peak emerging after the void volume contained 17.8 mg per ml, and this preparation was used for the enzyme assays. Enzyme preparations from the Neurospora methionineless mutants were made as described previously (1).

Enzyme Preparation from Yeast—One pound of yeast was crumbled and was dropped into 2 liters of liquid nitrogen. The frozen yeast was allowed to thaw, and the slurry was suspended in 450 ml of 0.3 M K₂HPO₄ at 4°. The pH was adjusted to 8.6 by the slow addition of concentrated NH₄OH with stirring. The mixture was stirred for 2 hours and centrifuged (Servall centrifuge, GSA rotor) at 8000 rpm for 1 hour. The supernatant liquid (600 ml) was removed and treated exactly as the Neurospora extract above, through the protamine sulfate and ammonium sulfate fractionations and the Bio-Gel chromatography. The final preparation contained 12.5 mg per ml of protein.

Enzyme Preparation from E. coli—Three liters of C medium were inoculated with 100 ml of seed culture of E. coli KB, and the culture was allowed to grow 12 hours with forced aeration. The cells (approximately 5 x 10⁹ per ml) were collected by centrifugation for 1 hour at 8000 rpm, washed with the sodium pyrophosphate buffer (0.06 M, pH 8.0), and centrifuged again. The pellet was suspended in 10 ml of the same buffer, and the cells were disrupted in a French pressure cell. One milliliter of deoxyribonuclease (50 μg per ml) was added, and the preparation was allowed to stand, with occasional stirring, until it was no longer viscous (approximately 1 hour); it was then centrifuged 10 min at 14,000 rpm (Servall centrifuge, rotor SS-34). The supernatant fluid was dialyzed 4 hours against the above buffer and then brought to 60% saturation with ammonium sulfate. The mixture was then centrifuged 1 hour at 14,000 rpm, and the precipitate was recovered and was diluted to 10 ml with the sodium pyrophosphate buffer (6 x 10⁻⁴ M, pH 8.0). The solution was chromatographed on Bio-Gel P 10 as described above for the Neurospora enzyme. The final preparation used for the enzyme assays contained 36.6 mg per ml of protein.

Enzyme Assays—The standard reaction mixture (for enzyme preparations from all three organisms) contained, in a final volume of 1 ml of potassium phosphate buffer (0.1 M, pH 7.3) in glass-stoppered centrifuge tubes, 0.188 μmol of pyridoxal phosphate, 10 μmoles of substrate (L-homoserine or its derivative), and 20 μmoles of sodium sulfide, together with the enzyme. For inhibition studies, L-methionine was added simultaneously with substrate.

Isolation of Reaction Product—Homocysteine (as its oxidized form, homocystine) was isolated from large scale reaction mixtures by the method described previously (1). The enzyme preparations from Neurospora and yeast were incubated with 190 μmoles of O-acetyl-L-homoserine, 400 μmoles of sodium sulfide, 1.88 μmoles of pyridoxal phosphate, and ε-amino (70 mg of the Neurospora protein; 50 mg of the yeast protein), in a final volume of 12 ml of potassium phosphate buffer (0.1 M, pH 7.3). A similar large scale reaction was carried out with E. coli enzyme with 190 μmoles of O-succinyl-L-homoserine and 146 mg of protein. The isolated crystalline product from each...
Table I
Homocysteine synthetase activity from Neurospora, yeast, and E. coli
Standard reaction mixtures and conditions, as described in "Methods," were used.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Specific activity of the enzyme (milliunits/mg protein)</th>
<th>Homocysteine (nitroprusside assay)</th>
<th>Homocysteine (ninhydrin assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurospora</td>
<td>O-Acetylhomoserine 0.1</td>
<td>18.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Yeast</td>
<td>O-Acetylhomoserine 0.1</td>
<td>30.0</td>
<td>0.3</td>
</tr>
<tr>
<td>E. coli</td>
<td>O-Succinylhomoserine 0.1</td>
<td>0.3</td>
<td>11.9</td>
</tr>
</tbody>
</table>

* Milliunit is that amount of enzyme which forms 1 mmole of homocysteine per min at 37°.

Table II
Isolation of homocysteine from homocysteine synthetase reactions

The large scale synthetase reactions were carried out and the homocysteine product isolated as outlined in "Methods."

<table>
<thead>
<tr>
<th>Organism</th>
<th>Substrate</th>
<th>Homocysteine (nitroprusside assay)</th>
<th>Homocysteine (ninhydrin assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurospora</td>
<td>O-Acetylhomoserine</td>
<td>22</td>
<td>19.2</td>
</tr>
<tr>
<td>Yeast</td>
<td>O-Acetylhomoserine</td>
<td>35</td>
<td>33.6</td>
</tr>
<tr>
<td>E. coli</td>
<td>O-Succinylhomoserine</td>
<td>37</td>
<td>37.2</td>
</tr>
</tbody>
</table>

* Calculated for the total reaction mixture from an assay on an aliquot from the mixture just prior to termination of the reaction.

Table III
Kinetics of homocysteine synthetases

<table>
<thead>
<tr>
<th>Organism</th>
<th>Substrate</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurospora</td>
<td>Homoserine</td>
<td>$5.5 \times 10^{-4}$ M</td>
<td>0.2 $\times 10^2$</td>
</tr>
<tr>
<td>Yeast</td>
<td>O-Acetylhomoserine</td>
<td>$4.5 \times 10^{-3}$ M</td>
<td>11.1 $\times 10^2$</td>
</tr>
<tr>
<td>Yeast</td>
<td>Homoserine</td>
<td>$6.6 \times 10^{-4}$ M</td>
<td>0.8 $\times 10^2$</td>
</tr>
<tr>
<td>E. coli</td>
<td>O-Acetylhomoserine</td>
<td>$5.5 \times 10^{-3}$ M</td>
<td>18.0 $\times 10^2$</td>
</tr>
<tr>
<td>E. coli</td>
<td>Homoserine</td>
<td>No reaction</td>
<td>7.1 $\times 10^2$</td>
</tr>
<tr>
<td>E. coli</td>
<td>O-Succinylhomoserine</td>
<td>3.9 $\times 10^{-3}$ M</td>
<td>7.1 $\times 10^2$</td>
</tr>
</tbody>
</table>

through the conversion of either substrate to the corresponding N-acetyl derivative would not have been apparent with the ninhydrin spray, quantitative tests on the reaction mixtures showed no decrease in the ninhydrin-positive material after 60 min of incubation.

Table I presents the specific activity of homocysteine synthetase for Neurospora, Yeast, and E. coli with either L-homoserine, O-acetyl-L-homoserine, or O-succinyl-L-homoserine. It may be observed that the specific activity of the Neurospora enzyme with O-acetylhomoserine is some 40 times that obtained with homoserine and some 25 times that obtained with O-succinylhomoserine. In the case of the yeast enzyme the specific activity with O-acetylhomoserine is 20 times that with homoserine and over 100 times that obtained with O-succinylhomoserine. The E. coli enzyme showed no activity with homoserine and only partial activity with O-acetylhomoserine, whereas incubation with the O-succinylated derivative produced a 40-fold increase in the specific activity. The enzyme preparations from all three organisms failed to show activity with phosphoserine or phosphohomoserine as substrate and, furthermore, no activity could be detected in a reaction mixture containing homoserine, adenosine triphosphate magnesium, and sodium sulfide. An enzyme preparation made from E. coli B (5 g of lyophilized cells, Worthington Biochemical Corporation) failed to show any activity for all the substrates, including homoserine and serine, and it may well be that the above activities can only be obtained from freshly grown cells.

Isolation of Reaction Product—Homocysteine (as homocysteine) was isolated and identified from the homocysteine synthetase reactions for each organism with its optimum substrate as described in "Methods." Paper chromatography of the isolated crystalline product, along with authentic L-homocysteine, followed by development of the chromatograms with ninhydrin spray reagent or chloroplatinic acid reagent, showed only one sulfur-containing compound present. This compound had the correct $R_f$ value for homocysteine. Table II indicates that there is close correspondence between the amount of crystalline homocysteine isolated (determined both by weight and by estimation of the ninhydrin reaction) and the amount of sulfhydryl material that was presumed to be homocysteine from the nitroprusside assay. This is confirmation that the major product of the reaction is homocysteine, and that other sulfhydryl-
containing compounds are not contributing to the results obtained from the nitroprusside assay.

$K_m$ and $V_{max}$ Values—Table III indicates the $K_m$ and $V_{max}$ values obtained for the enzyme preparations from the three organisms with their respective optimum substrates. It may be observed that the values for $K_m$ for each reaction are some 10-fold greater when the "activated" forms of homoserine are used as substrate. The $V_{max}$ values reflect the specific activity values given in Table I, and indicate the greatly increased velocity of the reactions when O-acetylhomoserine is the substrate for the enzyme preparations from Neurospora and yeast.

End Product Feedback Inhibition by Methionine—Table IV shows that L-methionine inhibits homocysteine synthetase activities from Neurospora and yeast when the acyl derivatives of homoserine are used as substrates. $K_i$ values are also listed, and, from a plot of $1/v$ versus $i$ (17), the inhibitions appear to be noncompetitive in each case. No inhibition by methionine on the enzyme activity from E. coli was observed.

### Table IV

**Inhibition of homocysteine synthetases by L-methionine**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Substrate</th>
<th>L-Methionine concentration</th>
<th>Inhibition</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurospora</td>
<td>O-Acetylhomoserine, 2.5 X 10^{-2} M</td>
<td>1</td>
<td>4</td>
<td>$2.15 \times 10^{-4}$ M</td>
</tr>
<tr>
<td></td>
<td>O-Acetylhomoserine, 5 X 10^{-2} M</td>
<td>1</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Yeast</td>
<td>O-Acetylhomoserine, 2.5 X 10^{-2} M</td>
<td>1</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O-Acetylhomoserine, 5 X 10^{-2} M</td>
<td>1</td>
<td>16</td>
<td>$2.4 \times 10^{-3}$ M</td>
</tr>
<tr>
<td>E. coli</td>
<td>O-Succinylhomoserine, 2.5 X 10^{-2} M</td>
<td>1</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O-Succinylhomoserine, 5 X 10^{-2} M</td>
<td>1</td>
<td>&lt;1</td>
<td></td>
</tr>
</tbody>
</table>

**Table V**

**Cysteine synthetase activity from Neurospora, yeast, and E. coli**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Specific activity of the enzyme</th>
<th>L-Series</th>
<th>O-Acetyl-L-serine</th>
<th>milliunits/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurospora</td>
<td></td>
<td>2.6</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Yeast</td>
<td></td>
<td>0.4</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td>&lt;0.01</td>
<td>12.6</td>
<td></td>
</tr>
</tbody>
</table>

**Discussion**

The data presented here clearly indicate that the preferred substrates for homocysteine synthetase from *Neurospora*, yeast, and *E. coli* are "activated" forms of homoserine. Thus, it is presumed that these derivatives of homoserine are ener-
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Our results with *E. coli*, which indicate the use of O-acetyl-L-serine for cysteine synthetase activity, corroborate those of others (11), who have, in addition, reported that two enzymatic steps are involved, namely, the acetylation of serine by acetyl-CoA followed by the sulfhydration to cysteine. On the other hand, the enzymatic synthesis of cysteine by *E. coli* with L-serine itself and sulfide has been demonstrated (12), and cysteine synthetase with L-serine as substrate has been reported to occur in yeast (19), spinach (20), beets (21) and *Neurospora* (1, 22).

The data presented here do not conflict with our previous conclusions regarding methionine biosynthesis, i.e. that cysteine and homocysteine can be synthesized de novo via separate routes which do not involve the formation or cleavage of cystathionine, and that the principal functions of that compound are storage and regulation (1, 23-25). Support for this point of view has come from the demonstration of the synthesis of cysteine and homocysteine from O-acetylserine and O-acetylhomoserine, respectively, with sulfide by enzyme extracts of spinach, in which the possibility of the intermediary formation of cystathionine was eliminated by studies of isotope competition. It was suggested that since crude extracts of spinach convert O-acetylhomoserine to S-adenosylhomocysteine, at a rate at least 10 times that of its conversion to cystathionine, the direct sulfhydration pathway may play a key role in the synthesis de novo of homocysteine (7).

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