O-ALKYLHOMOSERINE SYNTHESIS FROM O-ACETYLHOMOSERINE AND ALCOHOL

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Received September 14, 1970

SUMMARY

O-Alkylhomoserines are synthesized by an enzyme of Corynebacterium acetophilum A51 from O-acetylhomoserine and alcohols. The mutant strain $me^{-}74$ which cannot form O-ethylhomoserine from homoserine, ethanol, ATP and Mg^{++} and O-acetylhomoserine from homoserine and acetyl-CoA, forms O-ethylhomoserine from O-acetylhomoserine and ethanol. Purified enzyme from this organism synthesizes O-alkylhomoserines corresponding to the alcohols added from O-acetylhomoserine and ethanol, n-propanol, n-butanol or methanol, activities decreasing in the above order. Labeled ethanol is incorporated into O-ethylhomoserine in the presence of O-acetylhomoserine. O-Succinylhomoserine, phosphohomoserine and homoserine cannot replace O-acetylhomoserine. Moreover, ATP, Mg^{++} and pyridoxal-5'-phosphate are not required for the enzyme reaction.

We isolated O-ethylhomoserine, O-propylhomoserine and O-butylhomoserine as the first known naturally occurring ether amino acids from culture media of Corynebacterium ethanolaminophilum E17, containing ethanol, n-propanol and n-butanol, respectively(1,2). Later, O-methyl-, and O-pentylhomoserines were obtained by incubating methanol or n-pentanol and homoserine with cell suspensions of the organism(3).

O-4-Hydroxybutyl-, and O-3-hydroxybutyl-homoserines were also formed from 1,4-butanediol and 1,3-butanediol by cultures of bacteria utilizing 1,4-butanediol or 1,3-butanediol(4). Moreover, O-ethylhomoserine was found to be formed by many strains of Corynebacterium, Brevibacterium, Bacillus, Mycobacterium, Nocardia, Streptomyces(5), Pseudomonas(6), Clostridium(7) and yeast(8). We also found that an O-alkylhomoserine was involved in the biosynthesis of methionine from homoserine in Corynebacterium(9) and that alkylhomoserines were synthesized from homoserine and alcohols by a cell free extract of Corynebacterium acetophilum A51, the reaction requiring ATP, Mg^{++} and α-keto acid(10). The formation of O-alkylhomoserine was induced by alcohols and repressed by methionine and the enzyme involved was also inhibited by methionine(11). Recently, O-acetylhomoserine was shown by Nakayama et al.(12) to be an intermediate in methionine biosynthesis in a certain strain of Arthrobacter. Accordingly, we studied the role of O-acetylhomoserine in O-alkylhomoserine synthesis and found that O-acetylhomoserine was converted to O-alkylhomoserine in the presence of alcohols.

Corynebacterium acetophilum A51 and its mutant strain, $me^{-}74$, which is incapable of forming O-ethylhomoserine from homoserine and ethanol(9), were used. The abilities of cell free enzyme preparations from the two strains to synthesize O-acetylhomoserine and O-ethylhomoserine from homoserine and acetyl-CoA in the presence of ethanol and O-ethylhomoserine from O-acetylhomoserine and ethanol are shown in Table 1.

Both the wild and mutant strain formed O-ethylhomoserine from O-acetylhomoserine. The wild strain could also form O-acetylhomoserine from homoserine and acetyl-CoA but strain

me⁻74 could not. Thus, the reaction from homoserine to O-acetylhomoserine which has been shown to occur in Neurospora (13) and Saccharomyces(14), seems to be blocked in strain me⁻74 while homoserine and ethanol appear to be converted to O-ethylhomoserine through O-acetylhomoserine in the wild strain.

A crude enzyme preparation for O-alkylhomoserine synthesis from O-acetylhomoserine and alcohols obtained from the wild strain grown on ethanol medium was purified by fractionation with ammonium sulphate and then adsorption on a calcium phosphate cellulose gel column, followed by fractionation on Sephadex and DEAE cellulose columns. In this way the enzyme was purified about 70 fold. The optimum pH for the reaction was 7.5. ATP, Mg^{++} and pyridoxal-5'-phosphate were not required for this reaction. Only one absorption peak at 290 mμ was observed. In addition to ethanol, methanol, n-propanol and n-butanol were effective for alkylhomoserine synthesis(Table 2). Labeled O-ethyl-, O-propyl-, O-butyl- and O-methyl-homoserines were formed from ethanol, n-propanol, n-butanol and methanol, respectively, in the presence of labeled O-acetylhomoserine, the activities decreasing in the above order. The radioactive product from labeled O-acetylhomoserine and ethanol was identified to be O-ethylhomoserine by recrystallization with carrier. No O-alkylhomoserine was formed in the absence of alcohols. Acetaldehyde could not replace ethanol. α-Keto acid was not required for the reaction although previously α-keto acid was found to be utilized by the dialyzed enzyme preparation for O-ethylhomoserine synthesis from homoserine and alcohols in the presence of ATP and Mg^{++}(10). The effect of α-keto acid may be due to the formation of acetyl-CoA, resulting from addition of the keto acid.

Table 1

O-Acetylhomoserine and O-ethylhomoserine synthesis by crude enzyme extracts of the wild and mutant strain me⁻74 of Corynebacterium acetophilum A51.

	Additions to reaction mixture	OAH formed nmole/mg protein	OEH formed nmole/mg protein
Wild	[3,4-^{14}C]DL-Homoserine, ethanol, ATP, Mg^{++}	1.4	4.3
	[3,4-^{14}C]DL-Homoserine, ethanol, acetyl-CoA	28.9	7.1
	[3,4-^{14}C]O-Acetyl-DL-homoserine, ethanol	-	42.1
me⁻74	[3,4-^{14}C]DL-Homoserine, ethanol, ATP, Mg^{++}	0	0
	[3,4-^{14}C]DL-Homoserine, ethanol, acetyl-CoA	0	0
	[3,4-^{14}C]O-Acetyl-DL-homoserine, ethanol	-	38.3

*The abbreviations used are: OAH, O-acetylhomoserine; OEH, O-ethylhomoserine.

Wild and mutant strains were grown in shaking culture for 3 days at 30°C in 100 ml of medium containing 1% ethanol, 0.1% Bacto vitamin free casamino acids, 0.1% urea, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.001% NaCl, $MnCl_2 \cdot 4H_2O$ and $FeCl_3 \cdot 6H_2O$. The pH was adjusted to 7.4. About 1g wet weight of cells was harvested from 400 ml of cultures, washed twice with 0.05M phosphate buffer(pH 7.5) and suspended in 5 ml of the same buffer. The suspension was disintegrated in a Braun cell homogenizer at 4,000 rpm for 5 min. After centrifugation of the extract for 10 min at 11,000×g, the supernatant was centrifuged again for 1 hr at 77,000×g at 5°C. The supernatant was used as the enzyme preparation. Its protein concentration was about 30 mg/ml by the Lowry method. The assay mixtures contained, in 0.1 ml: 50 µl enzyme solution, 10 µmoles potassium phosphate buffer, pH 6.5, 0.5 µmole ethanol and other components. 0.1 µmoles of [3,4-^{14}C]DL-homoserine(1×10^6 cpm) and [3,4-^{14}C]O-acetyl-DL-homoserine(8×10^4 cpm), 0.5 µmole of acetyl-CoA, 0.05

μmole of ATP and 0.04 μmole of $MgCl_2 \cdot 6H_2O$ were used. Duplicate tubes were incubated for 20 min at 30°C. Reactions were stopped by addition of 10 μl of 0.04 N $Ba(OH)_2$ and 10 μl of 0.04 N $ZnSO_4 \cdot 7H_2O$ to remove protein. The mixture was made alkaline and O-acetylhomoserine was converted to N-acetylhomoserine as described previously(13). The supernatant was obtained by centrifugation at 2500 rpm for 10 min, and an 80 μl aliquot was spotted on paper. The papers were developed with the solvent, n-butanol-acetic acid-water (4 : 1 : 5, v/v/v). O-Ethylhomoserine (Rf 0.53) and N-acetylhomoserine (Rf 0.65) were extracted separately from the paper and dried on planchets for measurement of radioactivity. The activity was measured with an end-window gas-flow apparatus(Aloca DC 1 type).

Table 2

Synthesis of labeled O-alkylhomoserines from [3,4-^{14}C]O-acetylhomoserine and alcohols by purified enzyme.

	O-Alkylhomoserine formed	
	Compound	Amount μmole/mg protein
[3,4-^{14}C]O-acetyl-DL-homoserine		0
[3,4-^{14}C]O-acetyl-DL-homoserine plus methanol	*OMH	0.4
[3,4-^{14}C]O-acetyl-DL-homoserine plus ethanol	*OEH	2.6
[3,4-^{14}C]O-acetyl-DL-homoserine plus n-propanol	*OPH	2.2
[3,4-^{14}C]O-acetyl-DL-homoserine plus n-butanol	*OBH	0.9

The reaction mixture contained, in 0.1 ml, purified enzyme containing 0.05 mg of protein, 10 μmole of potassium phosphate buffer, pH 7.5, 0.1 μmole of [3,4-^{14}C]O-acetyl-DL-homoserine (8×10^4 cpm) and 0.5 μmole of alcohols. The incubation time was 20 min. The labeled O-alkylhomoserine formed was extracted from paper chromatograms and assayed by the same or a similar method to that described in Table 1. The Rf values of OMH, OEH, OBH and OBH are 0.45, 0.53, 0.62 and 0.74, respectively with the same solvents used in Table 1. *The abbreviations

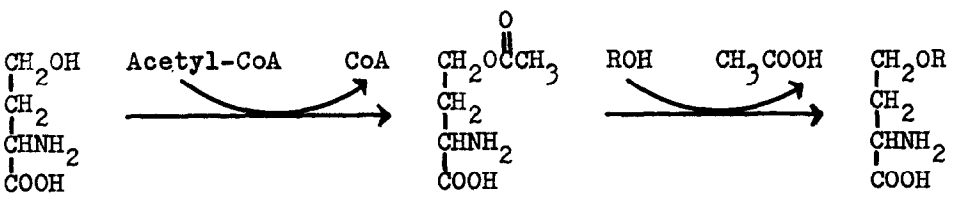
used are: OMH, O-methylhomoserine; OEH, O-ethylhomoserine; OPH, O-propylhomoserine; OBH, O-butylhomoserine.

Table 3

Incorporation of [2-^{14}C]ethanol into O-ethylhomoserine in the presence of O-acetylhomoserine by purified enzyme.

	O-Ethylhomoserine formed μmole/mg protein
[2-^{14}C]ethanol	0
[2-^{14}C]ethanol plus O-acetyl-DL-homoserine	1.4
[2-^{14}C]ethanol plus O-succinyl-DL-homoserine	0
[2-^{14}C]ethanol plus L-homoserine	0
[2-^{14}C]ethanol plus phosphohomoserine	0

The reaction mixtures contained, in 0.1 ml, 10 μmole of potassium phosphate buffer, pH 7.5, 0.1 μmole of O-acetylhomoserine and 0.5 μmole of [2-^{14}C]ethanol(8×10^5 cpm). The incubation time was 20 min. O-Ethylhomoserine formed was assayed as described in Table 1.



R = CH_3, C_2H_5, C_3H_7, C_4H_9.

Scheme 1. Pathways of biosynthesis of O-alkylhomoserines.

Labeled O-ethylhomoserine was synthesized from [2-^{14}C]ethanol and O-acetylhomoserine. O-Succinylhomoserine could not replace O-acetylhomoserine(Table 3). With strain me$^-$74, O-ethylhomoserine could be utilized for the growth in

place of methionine but O-succinylhomoserine could not(9). Another experiment showed that O-acetylhomoserine could replace methionine as well as O-ethylhomoserine for this mutant strain (Seto and Harada, Unpublished). O-Ethylhomoserine was not formed from [2-^{14}C]ethanol and homoserine or phosphohomoserine by this purified enzyme(Table 3).

From these results, the following pathway (Scheme 1) was proposed for the biosynthesis of O-alkylhomoserine from homoserine and alcohols. A reaction in which an ether bond is formed from an ester bond has very recently been demonstrated (15) in the synthesis of 1-O-alkyldihydroxyacetone phosphate from a long chain alcohol and acyldihydroxyacetone phosphate. The enzyme for the synthesis of O-alkylhomoserines from O-acetylhomoserine and alcohols is new and is of importance in that it is involved in synthesis of an ether bond and also in methionine biosynthesis and alcohol metabolism.

ACKNOWLEDGEMENT

We would like to thank Mr. T. Miwa for his valuable assistance.

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