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Dissimilation of Methionine by a Demethiolase of *Aspergillus* Species[1]

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Enzyme preparations obtained from the mycelium of *Aspergillus* species broke down methionine by co-dissimilation. The deaminase and demethiolase activities of crude extracts were increased 100-fold by precipitation with $(NH_4)_2SO_4$ and column chromatography on diethylaminoethyl cellulose. The enzyme acted on D-methionine but not on L-methionine. The enzyme was labile: it was inactivated by oxygen and ascorbic acid but ethylenediaminetetraacetic acid and mercaptoethanol preserved its activity. Enzyme activity decreased even at 4 and −30 C and was lost rapidly above 45 C. It was most rapid at 35 C and at *p*H 8.0 to 9.0. For the following reasons, it was concluded that deamination and demethiolation of methionine were effected by the same enzyme: both activities increased equally at each stage of purification; ammonia, methanethiol, and α-keto butyric acid were formed in amounts equivalent to the amount of methionine dissimilated; the K_m and optimal *p*H for formation of both keto acid and methanethiol were the same; both activities remained in the same fractions that were separated by electrophoresis and the activities were equivalent. The purified enzyme demethiolated α-keto methionine and α-hydroxy methionine and split the sulfur linkage of ethionine but did not cleave cystathionine. Few amino acids were deaminated. The enzyme was sensitive to some carbonyl and sulfhydryl reagents and was relatively insensitive to heavy metals other than Hg^{++}. The K_m was 1.3×10^{-3} to 1.5×10^{-3} M at *p*H 7.0. No requirement for cofactors was noted, and attempts to dissociate the enzyme, including dialysis with hydroxylamine, were unsuccessful.

Methanethiol has been reported to be the sulfur-containing product of breakdown of methionine by enzyme preparations of several bacteria. This was noted for dried cells of *Escherichia coli* and *Proteus vulgaris* (16) and for cell-free extracts of *E. coli* (15), cultures of *Pseudomonas* (6, 13), and a facultative anaerobe isolated from soil (11, 12). There is limited information about methionine dissimilation by fungal enzymes. Tsugo and Matsuoka (20) observed release of methanethiol from methionine by a filtrate of skim milk which had supported growth of *Penicillium caseicolum*. We reported degradation of methionine by whole cells (18) and by cell-free extracts (17) of *Aspergillus* species.

An exogenous source of energy (glucose) was required for demethiolation of the amino acid by pregrown fungal cell material (18). Studies with cell-free extracts of the fungus showed that an energy source [adenosine triphosphate (ATP)] served to neutralize an inhibitor of the enzyme which degraded methionine (17); ATP was required for demethiolation by a nondialyzed extract but not by a dialyzed one. The enzyme which deaminated and demethiolated methionine was constitutive, but more was present in cells which had been grown in the presence of the amino acid (17). It was noted also that the rates of both deamination and demethiolation by a crude cell-free extract were linear, that, generally, deamination was more rapid than demethiolation, and that deaminase activity was maximal at *p*H 7.0 to 9.0 and was reduced almost completely at *p*H 5.5. Furthermore, deaminase activity was nearly the same in atmospheres of oxygen, air, and nitrogen.

Herein we report the results of studies of purified cell-free extracts of the same fungus. These results include determinations of activity,

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specificity, and sensitivity of the enzyme to inhibitors. Also, products of the enzyme action are identified.

MATERIALS AND METHODS

Cultural methods. The fungus, *Aspergillus* species RS-la, was the same as that used previously (17, 18), as were the composition of the basal media, the general cultural methods, and the methods for determining methionine and the products of its decomposition.

Preparation of cell-free extracts and enzyme purification. The cell material which was used as the source of the enzyme was generally grown on a medium which contained 2.5% malt extract, 0.1% glucose, and 0.1% methionine. In some experiments, a chemically defined medium composed of mineral salts, 1.0% glucose, and 0.5% methionine was used. The medium was distributed in 100-ml amounts in twenty 250-ml Erlenmeyer flasks, sterilized, inoculated with a spore suspension of the fungus, and incubated for 4 days at 28 C on a rotary shaker. After incubation, the mycelium was filtered off, washed with distilled water, and suspended in 200 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.2). To this we added 60 to 80 g of glass beads, 20 μm in diameter, and the mycelium was homogenized by means of a Sorvall Omnimixer for 20 min at 14,000 rev/min, during which time the material was kept at 6 C or below by being held in an ice bath. Portions (80 ml) of the homogenate were held at 6 C during the 15-min period they were being disrupted by means of a colloid mill. The extracts were pooled and centrifuged for 15 min at 4 C in a Lourdes centrifuge.

The supernatant fluid, designated crude extract, was further purified by precipitation with $(NH_4)_2SO_4$ and column chromatography. Preliminary experiments indicated that most of the deaminase and demethiolase activities of the crude extract were recovered in material precipitated between 50 and 65% saturation with $(NH_4)_2SO_4$. The precipitation was done at 4 C as follows. The cell extract was brought to 50% saturation by adding solid $(NH_4)_2SO_4$, shaken for 15 min, and then centrifuged at 15,000 $\times$ *g* for 10 min. The solution beneath a floating layer of particulate material was removed, brought to 65% saturation with $(NH_4)_2SO_4$, shaken for 15 min, and centrifuged at 15,000 $\times$ *g*. The precipitate was dissolved in a small amount of 0.05 M Tris buffer (*p*H 7.2) which contained ethylenediaminetetraacetic acid (EDTA) and mercaptoethanol and was dialyzed with 7 liters of running buffer for at least 9 hr. By this procedure, the deaminase and demethiolase specific activities were increased 15 times.

Preliminary experiments indicated that oxygen and ascorbic acid had deleterious effects on the enzyme; solutions of the active material which had been precipitated with $(NH_4)_2SO_4$ turned brown and lost activity. These changes were prevented by 10^{-3} M EDTA and 10^{-3} M mercaptoethanol, which were included routinely in the 0.5 M Tris buffer.

Portions of the dialyzed enzyme solution (10 ml or less) were added to a column (1.9 by 21 cm) of diethylaminoethyl (DEAE) cellulose equilibrated with Tris buffer. The column was attached to a GM automatic fractionation collector, and the enzyme was eluted with a linear gradient of the Tris buffer (*p*H 7.2) starting with 0.05 M and ending with 0.4 M. The flux was 0.5 ml/min, and 10-ml fractions were recovered. The active preparations were dialyzed with deionized water, lyophilized, and held in an evacuated desiccator at -30 C until used. Under these conditions, activity was preserved for 3 months or more. Enzyme powder was dissolved in 0.05 M Tris buffer (*p*H 7.0) which contained 10^{-3} M EDTA and 10^{-3} M mercaptoethanol.

Electrophoresis of the purified and lyophilized product was done in a film of starch gel (0.25 by 7 by 14 cm) in contact with 0.5 M Tris buffer (*p*H 7.0) for 6 to 8 hr at 4 C with voltage maintained at 200 V. A thin strip of gel was cut from one edge of the sheet of gel and stained with amido schwartz 10B to locate the protein bands (3). The bands thus located were eluted from strips of the main sheet of gel by shaking in 0.05 M Tris buffer (*p*H 8.5) for 90 min.

Analytical methods. Protein was determined in crude extracts by the method of Lowry et al. (8) and in purified extracts by the method of Warburg and Christian (7). Conway dishes with plastic covers were used to determine methanethiol. A rubber stopper was sealed in a hole drilled in the cover. Cell extract, substrate (normally, 20 mM D-methionine), and supplementary material were added to the outer area of the bottom dish, 0.5 ml of 5% mercuric acetate was added to the central area, the top was sealed to the dish, and the unit was incubated at 28 C. The reaction was stopped by injecting 0.1 ml of 80% trichloroacetic acid through the rubber stopper with a hypodermic needle. The contents were mixed and left for 1 hr, after which the methanethiol trapped in the mercuric acetate was determined colorimetrically (19). When the method was checked with $(CH_3S)_2Pb$ dissolved in NaOH, recovery was greater than 99%. The procedure was standardized with $(CH_3S)_2Hg$ recrystallized from pyridine-water.

Conway units were used to determine ammonia which was trapped in 0.02 N H_2SO_4. In some experiments, enzyme reactions were carried out in small petri dishes, and the ammonia which was produced was measured by microdiffusion. Total keto acids were determined by Friedemann's method (5). Keto acids which were produced from methionine by purified extracts were determined by the method of McGee and Doudoroff (9). α-Keto-γ-methyl mercapto butyric acid (α-keto methionine) and α-keto-γ-hydroxy butyric acid were prepared by the enzyme method of Meister (10), modified in that crystalline D-amino acid oxidase was used instead of hog kidney acetone powder.

RESULTS

Enzyme purification. The active enzyme material which was obtained from the fungal extract by precipitation with $(NH_4)_2SO_4$ was purified further by column chromatography with DEAE cellulose as described above. The product is designated enzyme A. Figure 1 shows that the

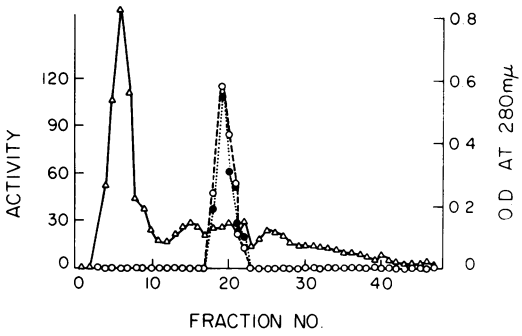

FIG. 1. *Chromatographic separation of fractions from ammonium sulfate precipitate of fungal extract. Symbols:* ○, *deaminase activity;* ●, *demethiolase activity;* △, *protein estimated by optical density at 280 nm. Activity is expressed as millimoles of* NH_3 *or* CH_3SH *released per minute per milligram of protein.*

same fractions recovered from the column had both deaminase and demethiolase activities. The same pattern of elution from the column was obtained with materials extracted from fungal cells grown on complex medium and on chemically defined medium. Results of purification of one preparation are included in Table 1. These results are typical of the many preparations which were made. Purification was about 100-fold; the ratio of the activities of deaminase and demethiolase remained approximately constant, and there was no separation of deaminase and demethiolase.

Enzyme A was subjected to electrophoresis on starch gel in an attempt to separate the enzymes responsible for the two activities and to purify the enzyme further. During electrophoresis both deaminase and demethiolase activities remained together in the material contained in the slowest moving bands, and they were quantitatively equivalent (Fig. 2). The same results were obtained in another experiment in which 0.05 M borate buffer (*p*H 8.2) was substituted for the 0.05 M Tris buffer (*p*H 7.0). The rates of both deamination and demethiolation by enzyme A were almost identical and were constant for at least 30 min (Fig. 3).

Saturation curves were calculated from the activity obtained at the end of a 10-min period of incubation. The Michaelis constant was calculated by the Lineweaver-Burk double reciprocal plot measuring the reaction by both deamination and demethiolation. The values found were approximately the same, 1.33×10^{-3} and 1.53×10^{-3} M, respectively.

Identification of products. Ammonia was identified by the Nessler reaction as the product of deamination of D-methionine by enzyme A. Another product was methanethiol, which was volatile under acid conditions and which was identified by the red material formed by its reaction with *N*,*N*-dimethyl *p*-phenylene diamine on addition of an oxidizing agent. This product had the same absorption spectrum as the product of the reaction between lead methyl mercaptide and *N*,*N*-dimethyl *p*-phenylene diamine. When the amount of enzyme was not limiting, all of the sulfur of the added methionine was recovered as

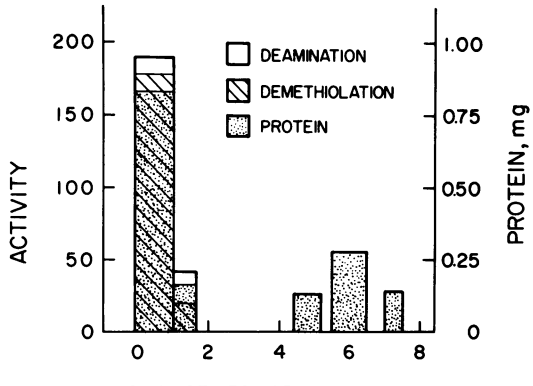

FIG. 2. *Electrophoresis of purified fungal enzyme on starch gel. Activity is expressed as millimoles of* NH_3 *or* CH_3SH *released per minute per milligram of protein.*

TABLE 1. *Purification of crude cell extract*

Fraction	Amt of protein	Activity		Purification		Yield (%)	
		Deamination[a]	Demethiolation[b]	Deamination	Demethiolation	Deamination	Demethiolation
	mg						
Crude extract	4,360	1.4	1.7				
Precipitated between 40 and 70% $(NH_4)_2SO_4$	241	19.2	19.2	14×	11×	76	62
Eluate of DEAE cellulose	3.9	175	167	125×	98×	11	9

[a] Millimoles of NH_3 released per minute per milligram of protein.
[b] Millimoles of CH_3SH released per minute per milligram of protein.

methanethiol, which indicated that there were no other sulfur products. The third product of action of the enzyme was α-keto butyric acid, which was identified by paper chromatography of its 2,4-dinitrophenyl hydrazone (Table 2) by use of several solvents (3). The 2,4-dinitrophenyl hydrazone had the same melting point (202 to 203 C) as the known derivative, and there was no depression of the melting point when a mixture of the two hydrazones was used. Infrared spectra of the 2,4-dinitrophenyl hydrazone of the product of enzyme action on methionine and of the known derivative (Nujol mulls) were the same (Fig. 4).

The enzyme failed to act on α-keto butyrate which accumulated as a product of methionine dissimilation. There was no loss of keto acid in solutions containing the enzyme preparation, α-keto butyrate, thiamine pyrophosphate, lipoic acid, and nicotinamide adenine dinucleotide.

Substrate specificity of the enzyme. Growing and replacement cultures of the fungus deaminated and demethiolated D- and L-methionine, but cell-free extracts and the purified enzyme acted on the D-isomer only, even though the fungus from which the enzyme was extracted had been cultivated on DL-methionine.

Deaminase activity of partially purified enzyme was restricted to a few compounds. Methionine, ethionine, and homocysteine were the only compounds of those tested which underwent appreciable deamination (Table 3). The following were not deaminated: alanine, α-amino butyric acid, aspartate, cystathionine, cysteine, glutamate, leucine, lysine, methioninol, serine, threonine, valine.

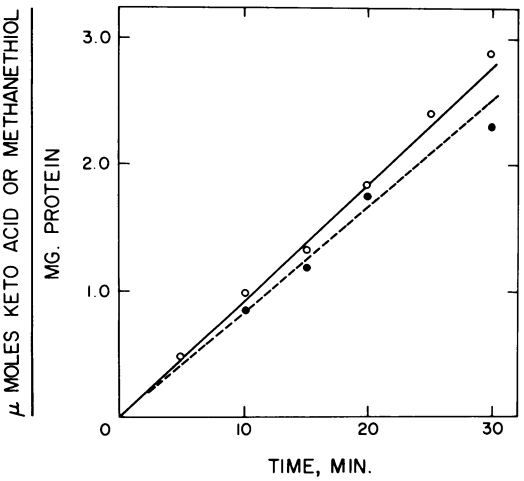

FIG. 3. *Rate of deamination* (○) *and demethiolation* (●) *of* D-*methionine by purified fungal enzyme.*

TABLE 2. *Chromatographic identification of α-keto butyric acid as a product of methionine dissimilation by the purified enzyme*

Solvent[a]	R_F of 2,4-dinitrophenyl hydrazone			
	α-Keto-butyrate	α-Keto-methio-nine	α-Keto-γ-hydroxy butyrate	Unknown
1	0.77			0.76
2	0.80		0.74	0.80
3	0.69	0.88		0.68
4	0.84		0.70	0.84
5	0.78			0.78

[a] Solvents: 1, *t*-amyl alcohol–1-propanol–concentrated NH_4OH (65:5:30); 2, ethyl alcohol–concentrated NH_4OH–water (80:5:15); 3, *n*-butyl alcohol saturated with 3% NH_4OH; 4, *n*-butyl alcohol–ethyl alcohol–water (40:10:50); 5, 20% ethyl alcohol in *n*-butyl alcohol.

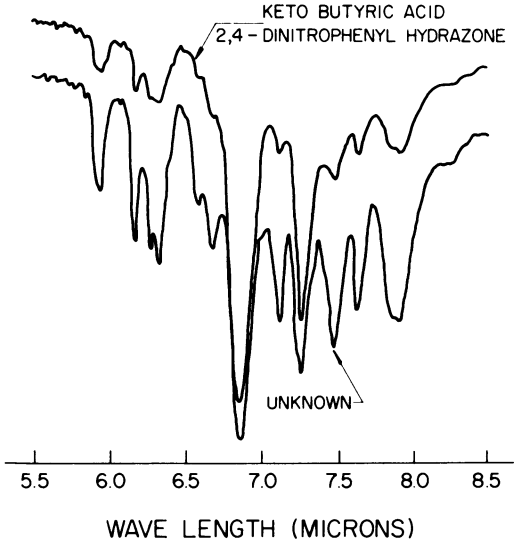

FIG. 4. *Infrared spectra of the 2,4-dinitrophenyl hydrazones of α-keto butyric acid and of the keto acid produced by action of purified fungal enzyme on* D-*methionine.*

TABLE 3. *Deamination by purified enzyme*

Substrate[a]	Activity relative to methionine[b]
	%
Methionine	100
Ethionine	89
Homocysteine	43
Homoserine	27

[a] Concentration, 10 mM.

[b] Calculated from micromoles of NH_3 per minute per milligram of protein.

The enzyme liberated methanethiol from α-keto methionine and α-hydroxy-γ-methyl mercapto butyric acid (α-hydroxy methionine). A volatile compound which gave a test for mercaptan, probably ethanethiol, was liberated from ethionine. Cleavage of cystathionine, determined by the method of Flavin (4), did not occur.

Stoichiometry of methionine dissimilation. To 1.5 ml of 5 mM D-methionine, we added 1.5 ml of a solution of enzyme A (0.352 mg of protein per ml) in Tris buffer (*p*H 8.5). After incubation of this mixture for 35 min, samples of the solution were used to determine keto acid, amino groups, ammonia, and residual methionine by the modified method of McCarthy and Sullivan. The amount of methanethiol produced was determined by means of a Conway unit by using another solution which was prepared from 0.5 ml of the enzyme solution and 0.5 ml of 5 mM D-methionine. This solution was incubated for 35 min. The amounts of products were equivalent to the amount of the substrate transformed and methanethiol was the only sulfur-containing product of demethiolation under the conditions used (Table 4). Furthermore, both reactions were simultaneous or quasisimultaneous.

Factors affecting activity of the enzyme. The optimal *p*H for both deamination and demethiolation was between *p*H 8.0 and 9.0. Activity nearly ceased below *p*H 7.0 and above *p*H 9.5.

Activity increased slowly from 20 C to a maximum at 35 C. Activity decreased at higher temperatures, and the decrease was abrupt above 45 C. To determine the effect of high temperatures, conical centrifuge tubes containing enzyme A in 6-ml portions of Tris buffer (*p*H 8.5) were immersed in boiling water. As soon as the enzyme solution reached the desired temperature, the tube was transferred to an ice bath and kept there for 5 min. The solutions were then centrifuged, after which deaminase activity of both the supernatant fluid and the precipitate was determined. The precipitates had no activity. Heating to 50 and 60 C decreased activity 80 and 92%, respectively, and higher temperatures inactivated the enzyme completely.

The results of several experiments indicated that the activity of the crude extract of the fungus was not promoted appreciably by glucose, succinate, or ATP. After the crude extract had been dialyzed with a small volume of buffer, demethiolase activity was increased by ATP (Table 5) and by glucose, succinate, and other hydrogen donors (15). However, after prolonged dialysis or purification by precipitation with $(NH_4)_2SO_4$ and column chromatography, the enzyme was fully active in the absence of any energy source such as ATP.

Additional results indicated that neither deaminase nor demethiolase activity of enzyme A was increased by the following cofactors: flavine mononucleotide (FMN), flavine adenine dinucleotide (FAD), FMN or FAD plus crude catalase, adenosine diphosphate, pyridoxal phosphate with or without ATP, or reduced glutathione with or without adenosine monophosphate. Since lack of response to cofactors might have been due to failure to remove coenzymes during the purification process, an attempt was made to dissociate and remove coenzymes by the method of Nishimura and Greenberg (14). Enzyme A was dialyzed with 0.02 M hydroxylamine in 0.05 M Tris buffer which contained EDTA and mercaptoethanol. After the dialysis, the hydroxylamine was removed by dialysis with Tris buffer. The enzyme lost none of its activity by the treatment, from which it is concluded that the enzyme either required no pyridoxal phosphate or that, if required, the pyridoxal phosphate was firmly bound to the enzyme.

Negative results were obtained in an attempt

TABLE 4. *Stoichiometry of enzymatic dissimilation of methionine*

Item determined	Amt of protein (moles per min per mg)
Amino groups lost	0.247
Ammonia produced	0.230
Keto acid produced	0.266
Methionine lost	0.227
Methanethiol produced	0.233

TABLE 5. *Effect of ATP on demethiolation by preparations of the fungal enzyme*

Amt of ATP added (μmoles)	Activity[a]				
	Crude extract			Purified enzyme	
	Non-dialyzed	Dialyzed against 1 liter of buffer	Dialyzed against 7.5 liters of buffer	7-fold[b]	30-fold[c]
0	1.50	0.66	1.83	5.56	47.6
0.25	1.50			5.56	
0.5	1.50	1.15	1.85	5.50	47.6
1.0	1.50		1.80	5.50	42.5
1.5	1.60	0.82	1.75		
2.0	1.60		1.77	4.20	42.5
3.0		0.79			

[a] Millimoles of CH_3SH produced per minute per milligram protein.

[b] Enzyme purified by $(NH_4)_2SO_4$ precipitation.

[c] Enzyme purified by $(NH_4)_2SO_4$ precipitation and chromatography with DEAE cellulose.

to detect the presence of a flavine prosthetic group in the enzyme by ammonium sulfate precipitation at acid reactions (*p*H 3.3 to 4.5) to dissociate the enzyme.

Deaminase activity was sensitive to hydroxylamine, arsenite, and cyanide (Table 6). It was somewhat less sensitive to the other inhibitors tested. With the exception of mercuric ions, deaminase activity was relatively insensitive to heavy metals (Table 7). The common cations (Na^+, K^+, NH_4^+, Ca^{2+}, Mg^{2+}, Mn^{2+}) at a concentration of 10^{-2} M had insignificant effects on deaminase activity.

TABLE 6. *Effect of inhibitors on deaminase activity*

Inhibitor	Inhibition (%) at	
	10^{-2} M	10^{-3} M
Hydroxylamine	96	77
Sodium arsenite	76	51
Sodium cyanide	83	43
p-Chloromercuribenzoate[a]		39
Sodium iodoacetate[a]		38
Sodium azide		32
Semicarbazide	69	17

[a] Activity measured by Friedemann's double extraction method (5).

TABLE 7. *Effect of heavy metals on deaminase activity*

Metal ion (10^{-3} M)	Inhibition
	%
Hg^{2+}	65
Pb^{2+}	31
Fe^{3+}	19
Cu^{2+}	14
Al^{3+}	11

DISCUSSION

Breakdown of methionine by the fungal enzyme resulted in production of ammonia, methanethiol, and α-keto butyrate in amounts equivalent to the methionine transformed. Similar results were obtained with living cells of the fungus (18), but the results differed in that the cells acted on both D- and L-methionine, whereas the enzyme preparation demethiolated only the D-sterioisomer. Therefore, it is probable that the fungus contains a methionine racemase as was noted for *Pseudomonas* species by Kallio and Larson (6). Unlike the fungal enzyme, the bacterial enzymes of Wiesendanger and Nisman (21) and of Kallio and Larson (6) were specific for the L-isomer.

From the following evidence, it is concluded that the same enzyme effected both deamination and demethiolation of methionine. (i) During purification of the cell-free extract, both activities increased to almost the same extent. (ii) Materials with deaminase and demethiolase activities were not separated by electrophoresis. (iii) The optimal *p*H values for deamination and demethiolation were nearly identical. (iv) The Michaelis constants which were determined after deamination and demethiolation were practically the same. (v) The rates of both deamination and demethiolation were almost the same. (vi) The products of both deamination and demethiolation were stoichiometrically equivalent. Similar results have been reported for cell-free extracts of bacteria (6, 12, 13, 21). However, the demethiolating enzyme systems of some bacteria (6, 15, 21) require pyridoxal phosphate, whereas the purified fungal enzyme showed no requirement for cofactors. A source of energy was required by the crude fungal extract but not by the pure enzyme. From this it is concluded that a source of energy is required to inactivate an inhibitor of the enzyme system. Binkley (1, 2) indicated that crude cystathioninase requires ATP and that the pure enzyme does not require it. He concluded that the ATP is required to phosphorylate pyridoxal, which is dephosphorylated in some way in crude extracts.

The enzyme was highly specific. There was sulfur cleavage of methionine, α-keto methionine, α-hydroxy methionine, and ethionine, but not of cystathionine, cysteine, or homocysteine. Furthermore, of the 16 amino acids tested, there was appreciable deamination of only 3, namely, methionine, ethionine, and homocysteine. Activity of the enzyme was confined principally to aliphatic compounds having a 4-carbon chain and containing a sulfur atom and a carboxyl group.

The enzyme was relatively insensitive to sulfhydryl reagents; at 10^{-3} M, iodoacetate and *p*-chloromercuribenzoate inhibited only 40% and mercuric ions inhibited only 65%. The carbonyl inhibitors cyanide, semicarbazide, and hydroxylamine were strongly inhibitory at 10^{-2} M. It is possible that the enzyme contained pyridoxal phosphate, but, if this was the case, the pyridoxal phosphate was tightly bound to the enzyme because activity was not decreased by dialysis with hydroxylamine.

Results reported on the course of events in the dissimilation of methionine suggest that it is different with different microorganisms. Equivalent amounts of products of deamination and demethiolation were obtained with the purified fungal enzyme, which suggests that both reactions occurred simultaneously. However, de-

amination is more rapid than demethiolation by some crude fungal enzyme preparations (17). Similar results were obtained with a cell-free extract of a bacterium (13). The fungal enzyme demethiolated α-keto methionine, and this was also noted by Mitsuhashi (11) for a bacterial enzyme. Since the bacterial enzyme of Kallio and Larson (6) failed to demethiolate α-keto methionine, they presumed that deamination does not precede demethiolation. Miwatani, Omukai, and Nakada (13) reported that α-amino butyric acid did not appear as a product of methionine dissimilation by their bacterial enzyme. Furthermore, the enzyme did not deaminate α-amino butyric acid. This supports the idea of Kallio and Larson that deamination does not precede demethiolation. Other results (12), including the detection of α-amino butyric acid as a product of the action of a certain bacterial enzyme (15), suggest that methionine is demethiolated before it is deaminated. The reaction by the fungal enzyme and the reactions of some of the bacterial enzymes (6, 12, 13, 21) yield α-keto butyric acid as a final product. The fungal enzyme is less sensitive to several inhibitors than the bacterial enzymes (13, 15). Furthermore, the fungal enzyme has nearly the same activity in oxygen, air, and nitrogen (17), whereas the bacterial enzymes were either tested only in the absence of oxygen (6, 12) or were found to be more active under anaerobic than aerobic conditions (11, 13, 15). These results lead to the conclusion that several different enzymes having the capacity to dissimilate methionine are produced by the different microorganisms which transform the amino acid.

ACKNOWLEDGMENT

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