Some Properties of a β -Cyanoalanine-forming Enzyme of *Enterobacter* sp. 10-1[†]

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The properties of a purified β -cyanoalanine (β -CNAIa)-forming enzyme isolated from *Enterobacter* sp. 10-1 were studied.

It has been found previously that this enzyme catalyzed the formation of cysteine from *O*-acetyl-L-serine and sodium sulfide, as well as the formation of β -CNAIa from *O*-acetyl-L-serine and sodium cyanide.

The affinity of the enzyme for sodium sulfide in the formation of cysteine was one order of magnitude greater than that for sodium cyanide in the β -CNAIa formation. In addition, the optimum pH, pH- and thermal stability of β -CNAIa-forming activity were similar to those of cysteine-forming activity. The results of the studies indicated that the β -CNAIa-forming enzyme of *Enterobacter* sp. 10-1 was an *O*-acetylserine sulphydrylase catalyzing the formation of cysteine from *O*-acetyl-L-serine and sulfide. The solution of the enzyme was yellow and exhibited an absorption peak at 420 nm. The enzyme itself contained approximately 1 mol of pyridoxal phosphate per 34,000 g of protein.

Previously, we have studied the purification and physical properties of a β -cyanoalanine (β -CNAIa)-forming enzyme from *Enterobacter* sp. 10-1, which catalyzes the formation of L- β -CNAIa from *O*-acetyl-L-serine and sodium cyanide.¹⁾ This enzyme also catalyzes the formation of cysteine from *O*-acetyl-L-serine and sodium sulfide, and its cysteine-forming activity is 245-fold greater than that of its β -CNAIa-forming activity. From these results, the β -CNAIa-forming enzyme of *Enterobacter* sp. 10-1 seems to be an *O*-acetylserine sulphydrylase catalyzing the formation of cysteine.

In order to clarify whether or not this β -CNAIa-forming enzyme is *O*-acetylserine sulphydrylase, the properties of the purified β -CNAIa-forming enzyme have been studied. In the present paper, characteristics of the enzyme are compared with those of a known *O*-acetylserine sulphydrylase, a cysteine-forming enzyme, which had been purified from

Salmonella typhimurium.²⁾

MATERIALS AND METHODS

Chemicals. Except as noted otherwise, all chemicals were from Wako Pure Chemical Industries, Ltd. (Osaka), and were of certified reagent grade. β -Cyano-L-alanine (β -CNAIa), *O*-acetyl-L-serine, *N*-acetyl-DL-serine, *O*-phospho-L-serine and *S*-methyl-L-cysteine were obtained from Sigma Chemical Co. (St. Louis).

Preparation of the purified β -CNAIa-forming enzyme. The purified enzyme was prepared by the purification systems described in the previous paper.¹⁾

Enzyme assay. The β -CNAIa-forming activity was determined in a reaction mixture containing 10 μ mol of *O*-acetyl-L-serine, 5 μ mol of sodium cyanide, 200 μ mol of Tris-HCl buffer (pH 7.4) and the enzyme solution in a total volume of 1.0 ml. The reaction was carried out at 30°C for 5 min, and the amount of β -CNAIa synthesized was determined by paper chromatography. The unit of activity and specific activity of the enzyme were the same as those described in the previous paper.¹⁾

Cysteine-forming activity was determined in a reaction mixture containing 5 μ mol of *O*-acetyl-L-serine, 0.5 μ mol of sodium sulfide, 0.16 μ mol of EDTA, 32 μ mol of Tris-

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HCl buffer (pH 7.4) and the enzyme solution in a total volume of 0.2 ml. The reaction was carried out at 30°C for 5 min, and the amount of cysteine synthesized was determined according to the method described by Becker *et al.*²⁾ Again, the unit of activity and the specific activity of the enzyme were the same as were described previously.¹⁾

Other methods. Protein was assayed according to the method of Lowry *et al.*³⁾ with bovine serum albumin as the standard protein, or by measuring the absorbance at 280 nm. Pyridoxal phosphate was determined by the method of Wada and Snell.⁴⁾

RESULTS

Substrate specificity

The substrate specificity of the enzyme was investigated. According to the data presented in Table I, only *O*-acetyl-L-serine served as a substrate for both cysteine and β -CNAla formation. Formation of both cysteine and β -CNAla in the experiments described below indicated the presence of enzyme activity.

Effect of substrate and cosubstrate concentration on the enzyme activity

The affinity of the enzyme for the different substrates, *O*-acetyl-L-serine, sodium cyanide and sodium sulfide, was examined. As is

shown in Fig. 1, the apparent K_m 's for *O*-acetyl-L-serine in both the β -CNAla- and the cysteine-forming reactions were very close to one another, 6.7 mM and 8.3 mM, respectively. The apparent K_m for sodium cyanide was found to be 5.2 mM, while that for sodium sulfide was 0.5 mM, in their respective reactions. In addition, the affinity for sodium

TABLE I. SUBSTRATE SPECIFICITY OF THE β -CNAla-FORMING ENZYME

The β -CNAla- and cysteine-forming activities were assayed according to the methods described in the text, except for the substrate changes indicated.

Substrate	Relative activity (%)	
	β -CNAla formed	Cysteine formed
<i>O</i> -Acetyl-L-serine	100	100
<i>N</i> -Acetyl-DL-serine	0	0
<i>O</i> -Phospho-L-serine	0	0
<i>S</i> -Methyl-L-cysteine	0	0
DL-Alanine	0	0
L-Asparagine	0	0
L-Aspartic acid	0	0
L-Cysteine · HCl	0	0
Glycine	0	0
L-Threonine	0	0
L-Serine	0	0

FIG. 1. Effect of Substrate and Cosubstrate Concentrations on the β -CNAla-Forming Enzyme.

The β -CNAla-forming activity (A) and cysteine-forming activity (B) were assayed as described in MATERIALS AND METHODS except that the concentrations of *O*-acetyl-L-serine, sodium cyanide and sodium sulfide were changed.

FIG. 2. The pH Stability of the β -CNAla-Forming Enzyme.

The enzyme solution was incubated at 37°C for 60 min at the pH's indicated in the figure. The residual β -CNAla-forming activity (A) and cysteine-forming activity (B) were assayed according to the procedures described in MATERIALS AND METHODS. The buffers used in this experiment were: (—●—), acetate; (—▲—), phosphate; (—■—), Tris-HCl; (—○—), borate.

FIG. 3. Thermal Stability of the β -CNAla-Forming Enzyme.

The enzyme, dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM 2-mercaptoethanol, was incubated for 10 min at the temperatures indicated in the figure. The residual β -CNAla-forming activity (A) and cysteine-forming activity (B) were assayed as described.

FIG. 4. Effect of pH on Enzyme Activity.

The β -CNAla-forming activity (A) and the cysteine-forming activity (B) were determined as described, except for changes in pH indicated in figure. The buffers used were: (—●—), phosphate; (—▲—), Tris-HCl; (—■—), borate.

sulfide in the cysteine formation was one order of magnitude greater than that for sodium cyanide in the β -CNAla formation.

Stability

The enzyme was stable over a pH range of 6 to 10, when incubated for 60 min at 37°C (see Fig. 2). Moreover, as Fig. 3 shows, the enzyme was stable up to 60°C when incubated at pH 7.5 for 10 min. The stabilities of the cysteine- and the β -CNAla-forming activities toward changes in pH and temperature were found to be identical.

Effect of pH on enzyme activity

In order to examine the effect of pH on the cysteine- and the β -CNAla-forming activities, the reactions were carried out at various pH's from 7.0 to 11.0, as is shown in Fig. 4. The optimum pH of both activities was found between 9.5 and 10.0 (in both Tris-HCl and borate buffers).

Effect of enzyme inhibitors

The effects of some compounds, generally considered as inhibitors of enzyme activity, were examined, and the results are shown in

TABLE II. EFFECT OF INHIBITORS ON THE β -CNAIa-FORMING ENZYME

β -CNAIa- and cysteine-forming activities were determined by the assay methods described in the text, except for addition of certain reagents as inhibitors.

Inhibitor (1 mM)	Relative activity (%)	
	β -CNAIa formed	Cysteine formed
None	100	100
NaNO ₃	86	102
NaF	63	100
NaN ₃	97	89
Na ₂ HAsO ₄	96	—
Thiourea	81	66
α,α' -Dipyridyl	69	90
<i>o</i> -Phenanthroline	89	86
Monoiodoacetate	100	—
<i>N</i> -Ethylmaleimide	107	—
HgCl ₂	38	—
<i>p</i> CMB	31	60
<i>p</i> CMPSA	25	54
Mersalyl acid	38	—

Table II. Mercuric compounds such as HgCl₂, *p*CMB (*p*-chloromercuribenzoate), *p*CMPSA (*p*-chloromercuriphenyl sulfonic acid) and mersalyl acid appeared to inhibit the enzyme reaction. The effects of various metal ions on both enzyme activities were also examined. As can be seen in Table III, both enzyme activities were inhibited by some metal ions, such as Cd²⁺, Co²⁺, Ni²⁺ and Zn²⁺, and their inhibition rates were identical.

Absorption spectrum

The purified enzyme was intensely yellow, and its absorption spectrum exhibited a peak at 412 nm (see Fig. 5), which presumably indicates the presence of pyridoxal phosphate. The ratio of the absorbance at 280 nm to that at 412 nm was determined as 3.3.

Prosthetic group of the enzyme

In order to clarify the presence of pyridoxal phosphate or its derivatives in the enzyme, because of its yellow color, some additional studies were indicated. The effects of known inhibitors of enzymes containing pyridoxal phosphate as the prosthetic group were exam-

TABLE III. EFFECT OF METAL SALTS ON THE β -CNAIa-FORMING ENZYME

Both the β -CNAIa- and cysteine-forming activities were determined by the assay methods given in MATERIALS AND METHODS except for the addition of the compounds indicated.

Metal salt (1 mM)	Relative activity (%)	
	β -CNAIa formed	Cysteine formed
None	100	100
AgNO ₃	22	95
BaCl ₂	106	110
CaCl ₂	96	95
CdCl ₂	33	45
CoCl ₂	12	—
CuSO ₄	33	—
FeCl ₂	109	—
FeCl ₃	99	—
KCl	86	91
MgCl ₂	103	96
MnCl ₂	107	100
NaCl	94	92
NiCl ₂	10	—
PbCl ₂	86	—
SnCl ₂	107	—
ZnCl ₂	25	5

FIG. 5. Absorption Spectrum of the β -CNAIa-Forming Enzyme.

The absorption spectrum of the crystalline enzyme was measured with a Hitachi 124 spectrophotometer. The enzyme (1.88 mg of protein/ml) was first dialyzed against 50 mM potassium phosphate buffer (pH 7.5). The absorbance was measured using the dialysis fluid as a blank.

TABLE IV. EFFECT OF SOME COMPOUNDS ON THE β -CNAla-FORMING ENZYME

The β -CNAla- and cysteine-forming activities were assayed according to the methods described in MATERIALS AND METHODS, except that the compounds indicated were added in the given concentrations.

Compound	Concn. (mM)	Relative activity (%)	
		β -CNAla formed	Cysteine formed
Hydroxylamine·HCl	50	5	38
	20	36	53
	10	68	63
	5	110	75
	1	100	69
Hydrazine hydrate	50	—	—
	20	—	—
	10	105	—
	5	125	—
	1	109	97
Cycloserine	20	—	—
	10	—	88
	5	—	88
	1	88	100
Sodium borohydride	10	5	18
	1	7	41
Tris	600	9	—
None		100	100

ined. As shown in Table IV, these enzyme reactions were inhibited by relatively high concentrations of hydroxylamine and sodium borohydride, both of which are also inhibitors of pyridoxal phosphate-linked enzymes. Furthermore, pyridoxal phosphate added to the reaction mixture did not affect enzyme activity. However, when *O*-acetyl-L-serine was added to the enzyme, a marked change in the absorption spectrum was observed (see Fig. 6), and there appeared an absorption peak at 470 nm, which is probably due to complex-formation by the *O*-acetyl-L-serine and the enzyme-bound pyridoxal phosphate. From these results, the pyridoxal phosphate can be assumed to bind tightly to the enzyme protein. The amount of pyridoxal phosphate present in the enzyme was determined to be 0.75 mol per 1 mol of enzyme protein (molecular weight of 34,000).

DISCUSSION

In a previous paper,¹⁾ we have presented evidence that the β -CNAla-forming enzyme from *Enterobacter* sp. 10-1 (Strain 10-1) catalyzes the formation of cysteine from *O*-acetyl-L-serine and sodium sulfide in addition to that of L- β -CNAla, and that the cysteine-forming activity was 245-fold greater than the β -CNAla-forming activity. From these results,

FIG. 6. Spectral Changes of the β -CNAla-Forming Enzyme.

The spectra were recorded on a Hitachi 124 spectrophotometer. The protein concentration was 0.28 mg per ml in 50 mM potassium phosphate buffer (pH 7.5). Sodium sulfide (A), sodium cyanide (B) and *O*-acetyl-L-serine (C) were added to the given concentrations.

the β -CNAla-forming enzyme also seemed to be the cysteine-forming enzyme, *O*-acetylserine sulfhydrylase, which catalyzes the formation of L-cysteine.

In order to clarify the pathway of the β -CNAla-forming enzyme, its affinity for the substrates, *O*-acetyl-L-serine, sodium cyanide and sodium sulfide was examined. The apparent K_m 's for *O*-acetyl-L-serine in both cases were very close to one another, 6.7 mM for β -CNAla formation, and 8.3 mM for cysteine production. However, the apparent K_m for sodium cyanide was 5.2 mM, and that for sodium sulfide was 0.5 mM, in their respective reactions. The affinity for sodium sulfide in cysteine formation was one order of magnitude greater than that for sodium cyanide in β -CNAla formation. These results suggested that the β -CNAla-forming enzyme in *Enterobacter* sp. 10-1 was an *O*-acetylserine sulfhydrylase catalyzing the formation of cysteine from *O*-acetyl-L-serine and sulfide. In addition, the optimum pH for both the cysteine and β -CNAla activities was approximately 9.5, and they exhibited the same stability toward pH and temperature. Both of the enzyme activities

were inhibited to similar extents by some metal ions and by some known enzyme inhibitors.

On the other hand, the enzyme contained almost 1 mol of pyridoxal phosphate per 34,000 g of protein. When *O*-acetyl-L-serine was added to the enzyme, a marked change in the absorption spectrum was observed, with the appearance of a peak at 470 nm. Such an effect is probably the result of the formation of a complex between *O*-acetyl-L-serine and the enzyme-bound pyridoxal phosphate. Thus, after the formation of this complex, cyanide and some thiol groups seem to act as cosubstrates in place of sulfide, whose role had been described in a previous paper.¹⁾

The authors showed that the β -CNAla-forming enzyme of *Enterobacter* sp. 10-1 was an *O*-acetylserine sulfhydrylase. *O*-Acetylserine sulfhydrylase have been found in various organisms. Becker *et al.*²⁾ purified one such enzyme from *Salmonella typhimurium* as a homogeneous preparation, and studied its properties. In the present study, we have compared some properties of the *Enterobacter* enzyme with those of *S. typhimurium* enzyme described by Becker *et al.*²⁾ These comparisons

TABLE V. SOME PROPERTIES OF *O*-ACETYL SERINE SULFHYDRYLASE

Properties	<i>Enterobacter</i> sp. 10-1	<i>S. typhimurium</i>
Molecular weight	68,000 ^a (34,000 $\times$ 2) ^b	68,000 ^c (34,000 $\times$ 2) ^c
$S_{20,w}$	4.75S	4.5S
Isoelectric point (pH)	5.0	—
$E_{1\text{ cm}}^{1\%}$	8.7	12.1
B ₆ content (mol/34,000 g protein)	0.75	1.0
Substrate specificity	<i>O</i> -AS ^d	<i>O</i> -AS ^d
Cosubstrate specificity	Na ₂ S, NaCN, Methylmercaptan, Ethylmercaptan, Phenylmercaptan, 2-Mercaptoethanol.	Na ₂ S, Methylmercaptan.
K_m for <i>O</i> -AS (mM) ^d	8.3	5.0
for Na ₂ S (mM)	0.5	0.1
for NaCN (mM)	5.2	—
Cysteine-forming activity (units/mg protein)	711	1,100

^a By gel filtration.

^b By SDS-electrophoresis.

^c By equilibrium sedimentation.

^d *O*-AS (*O*-acetyl-L-serine).

TABLE VI. AMINO ACID COMPOSITION OF
O-ACETYL-SERINE SULFHYDRYLASE

Amino acid	Number of residues per subunit (MW 34,000) round off to the nearest integer	
	<i>Enterobacter</i> sp. 10-1	<i>S. typhimurium</i>
Lysine	24	26
Histidine	2	3
Arginine	11	14
Aspartic acid	24	26
Threonine	20	24
Serine	15	17
Glutamic acid	32	38
Proline	13	16
Glycine	28	33
Alanine	27	31
Valine	25	20
Methionine	2	5
Isoleucine	20	24
Leucine	27	32
Tyrosine	6	6
Phenylalanine	6	7
Cysteine	1	0.7
Tryptophan	3	5

are summarized in Table V, while Table VI shows the amino acid composition of these enzymes. From the results, it can be seen these two enzymes are similar in amino acid composition, absorption spectrum, molecular weight and affinity for *O*-acetyl-L-serine.

However, Becker *et al.*²⁾ did not study the β -CNAla-forming activity of the *S. typhimurium* enzyme. Thus, the present study is the first elucidation in a microorganism of an enzyme having β -CNAla-forming activity.

The main pathway of formation of β -CNAla in Strain 10-1 was presumed to be that the exogenous cyanide is incorporated rapidly into the cell, and in the cytoplasm it is combined with *O*-acetyl-L-serine to form β -CNAla by *O*-acetylserine sulfhydrylase. Although Strain 10-1 was found not to degrade β -CNAla, some microorganisms assimilate β -CNAla to form asparagine or aspartic acid. Therefore, the cyanide fixation pathway *via* β -CNAla is presumed to be a pathway to extend the carbon chain of a amino acid in microorganisms.

REFERENCES

- 1) H. Yanase, T. Sakai and K. Tonomura, *Agric. Biol. Chem.*, **46**, 355 (1982).
- 2) M. A. Becker, N. M. Kredich and G. M. Tomkins, *J. Biol. Chem.*, **244**, 2418 (1969).
- 3) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 4) H. Wada and E. E. Snell, *J. Biol. Chem.*, **236**, 2089 (1961).