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Beta-Cyanoalanine Synthase: Purification and Characterization

(pyridoxal-*P*/β-replacement-specific lyases/thiocyanate/isotopic α-hydrogen exchange)

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ABSTRACT **Beta-cyano-L-alanine synthase [L-cysteine hydrogen-sulfide-lyase (adding HCN), EC 4.4.1.9] was purified about 4000-fold from blue lupine seedlings. The enzyme was homogeneous on gel electrophoresis and free of contamination by other pyridoxal-*P*-dependent lyases. The enzyme has a molecular weight of 52,000 and contains 1 mole of pyridoxal-*P* per mole of protein; its isoelectric point is situated at pH 4.7. Its absorption spectrum has two maxima, at 280 and 410 nm. L-Cysteine is the natural primary (amino acid) substrate; β-chloro- and β-thiocyano-L-alanine can substitute for it. Some small aliphatic thiols can serve (with considerably lower affinity) instead of cyanide as cosubstrates for cyanoalanine synthase. The synthase is refractory to DL-cycloserine and D-penicillamine, potent inhibitors of many pyridoxal-*P*-dependent enzymes. Cyanoalanine synthase catalyzes slow isotopic α-H exchange in cysteine and in end-product amino acids; the rates of α-H exchange in nonreacted (excess) cysteine are markedly increased in the presence of an adequate cosubstrate; no exchange is observed of H atoms in β-position.**

Incorporation of labeled C from [^{14}C]cyanide into the carboxamide group of asparagine was demonstrated in seedlings of higher plants (certain Leguminosae; *Sorghum* sp.) (1, 2), and also in bacteria (3) and fungi (4). β-Cyano-L-alanine is an intermediate in the assimilation of cyanide (5). L-Serine and, more effectively, L-cysteine served as primary substrates for the enzyme, known as β-cyanoalanine synthase [L-cysteine hydrogen-sulfide-lyase (adding HCN), EC 4.4.1.9], which forms this intermediate in *Escherichia coli* (3) and in plant extracts (6). Hendrickson and Conn (7) purified β-cyanoalanine synthase [(CN)Ala synthase] 140-fold from mitochondrial acetone powder of blue lupine seedlings. As amino substrates their preparation used L-cysteine and (20 times less actively) *O*-acetyl-L-serine. It failed to utilize L-serine and various analogs of serine and cysteine, but catalyzed exchange of labeled HS between cysteine and H_2S, slow synthesis of L-cysteine from H_2S and *O*-acetylserine, and of *S*-methylcysteine from cysteine and methanethiol.

In recent years we made comparative studies of some vitamin-B_6-dependent lyases exclusively catalyzing replacement reactions of β-substituent in cysteine, serine, and related analogs (8, 9, 18, 19, 25). As a rule, these lyases, e.g., cysteine lyase (EC 4.4.1.10), serine sulfhydrase, and the identical or closely similar cystathionine β-synthase (EC 4.2.1.22), use aliphatic thiols as cosubstrates (replacing agents). It seemed of particular interest to extend our studies to (CN)Ala synthase, a β-lyase utilizing cyanide as the preferred cosubstrate.

Abbreviations: Pyridoxal-*P*, pyridoxal-5′-phosphate; (CN)Ala, β-cyano-L-alanine; (Cl)Ala, β-chloroalanine; (CNS)Ala, β-thiocyano-L-alanine; HS-EtOH, 2-mercaptoethanol; r.s.r., relative specific radioactivity.

In this paper, we present an improved purification procedure for (CN)Ala synthase, and report on some general and catalytic properties of the enzyme.

MATERIALS AND METHODS

Reagents and Instruments. All chemicals were preparations of highest purity available, namely: DEAE-Sephadex A-50 and Sephadex G-100 (Pharmacia, Uppsala); hydroxylapatite from Bio-Rad; pyridoxal-*P* (Reanal, Budapest); dansyl chloride (Merck); reference dansylamino acids (from "Serva"); ^3H_2O (61 mCi/ml), made in USSR. Aluminum hydroxide C_γ was prepared according to Willstätter and Kraut (27). We thank Dr. Drell (Calbiochem) for the gift of a sample of β-cyanoalanine.

Reagents and apparatus for analytical gel electrophoresis were supplied by Reanal; Ampholine buffers and equipment for isoelectrofocusing, by LKB Producter (Stockholm). Chromatographic and electrophoretic separations were performed on papers FN-4 and FN-16 ("Filtrak", German Democratic Republic). All buffer solutions used were Tris·HCl of pH 8.8, in the concentrations indicated.

Enzyme Activity Assays. (CN)Ala synthase activity assays were based on the rates of formation of end-products—H_2S, (CN)Ala, or thiocyanate. One unit is defined as the amount of enzyme yielding 1 μmol of product per min under the assay conditions; specific activity, as U/mg of protein.

Sulfide liberated from cysteine was assayed by spectrophotometry either (*1*) after conversion to methylene blue, as described in ref. 10 (an $A_{650\,nm}$ value of 1.0 corresponds to 0.65 μmol of sulfide in this assay), or (*2*) in the form of colloidal PbS solution, as described in ref. 9; in this procedure 0.36 μmol of PbS gave $A_{360\,nm} = 1.0$.

To locate (CN)Ala synthase in gel slabs after preparative electrophoresis, a strip of the gel was incubated 20 sec at 30° in 0.1 M Tris buffer containing L-cysteine and KCN (2.5 mM each) and 0.2 mM $Pb(OAc)_2$; appearance of a brown band revealed the position of the enzyme in the gel.

β-Cyanoalanine production was measured by incubation (30°, 10 or 20 min) of samples (0.5 ml) containing [^{14}C]KCN and amino substrate (2.5 mM each) and 20–30 mU of enzyme. One-tenth aliquots of the incubated samples, fixed by acidification to pH 5 (with acetic acid), were streaked on paper strips and subjected to high voltage electrophoresis for 40 min in acetic acid/formic acid/water (4:1:45) or to chromatography with butanol/formic acid/water (15:3:2) solvent. Thiocyanate was determined by the method of Sorbö (11); 0.85 μmol CNS^- per sample on spectrometry as the Fe^{3+} salt gave $A_{470\,nm} = 1.0$.

Amino acids on paper chromatograms were stained lightly with acetonic ninhydrin solution, and estimated quantita-

TABLE 1. *Purification of cyanoalanine synthase from blue lupine seedlings**

Enzyme fraction after	Protein, mg	Total activity, U (H_2S μmol/min)	Specific activity, U/mg
Extraction of mitochondrial acetone powder	2475	520	0.2
DEAE-Sephadex A-50	330	496	1.5
C_γ gel	116	404	3.5
Sephadex G-100	18.5	232	12.5
Hydroxylapatite	6.8	152	23.1
Preparative gel electrophoresis	2.9	126	43.5

* Assay: H_2S release from L-cystine + KCN (see *Methods*). Total activity in homogenate of lupine seedlings, 732 U; specific activity was 0.011 U/mg.

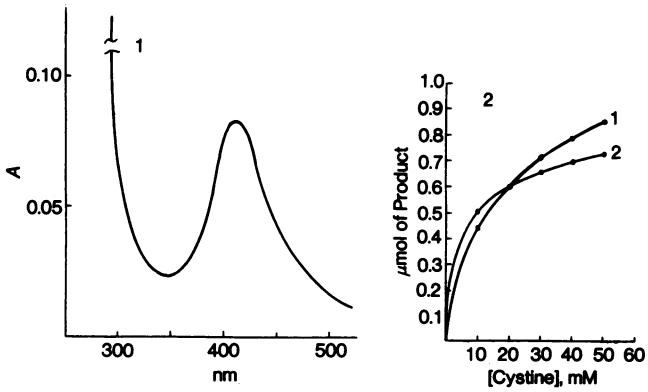

FIG. 1. Absorption spectrum of (CN)Ala synthase. Specific activity: 37.5 U/mg; 1.6 mg of protein per 1 ml of Tris buffer (0.1 M). Cuvette length 1 cm.

FIG. 2. Interaction of (CN)Ala synthase with L-cystine and KCN. Production of hydrogen sulfide (curve 1) and thiocyanate (curve 2) versus L-cystine concentration.

Ordinate: ΔH_2S and ΔCNS^- in μmol from 0 to 20 min.

tively by spectrophotometry as Cu^{2+}-ninhydrin complexes (12); cysteine was determined similarly after oxidation with performic acid (13) and electrophoretic separation of the cysteic acid.

Protein was estimated by spectrophotometry at 280 nm, or according to Lowry *et al.* (14). Analytical gel electrophoresis was performed as described by Davis (15), locating protein by staining with amido black, and enzymatic activity by detection of H_2S with $Pb(OAc)_2$ (see above).

Identification of N-Terminal Residue. High-purity enzyme was subjected to dansylation, followed by hydrolysis in 6 N HCl and identification of dansylamino acids by thin-layer chromatography (16). The only *N*-terminal detected was a glutamate residue.

Protium–Tritium Exchange was investigated in experiments similar to those reported in ref. 25, using Tris·HCl buffer in ^3H_2O diluted to specific radioactivity 1.5 to 5.0 × 10^5 cpm/μg-atom H. Samples contained L-cysteine (15 μmol), KCN (15 μmol) or 2-HS-EtOH (150 μmol), and 100–200 mU of enzyme. Incubated samples, acidified to pH 5 with HCl, were lyophilized with repeated addition of H_2O to completely remove volatile label. Radioactivity of nonvolatile tritiated compounds, separated on paper and eluted, was measured on an automatic scintillation counter (SL-40) and expressed as cpm/μg-atom H in 1 μmol of product (or as relative specific radioactivity in percent of specific radioactivity of the solvent water). To check for eventual exchange of H atoms in β-position, L-[β-^{3}H]Cysteine (Amersham) was incubated with KCN and the enzyme in nonlabeled buffer.

The Isoelectric Point of (CN)Ala synthase was determined by electrophoresis with Ampholine buffer mixture pH 3.0 → 10.0 in 110 ml columns (LKB) (17).

RESULTS

Purification of Cyanoalanine Synthase from Blue Lupine Seedlings. Mitochondrial acetone powder was made from etiolated 10-day-old lupine seedlings (4–7 kg), processed essentially as described in ref 7. The vacuum-dried white acetone powder was kept at −20°; all further operations were carried out at +4°.

Chromatography on DEAE-Sephadex. The acetone powder was dispersed in 0.05 M Tris buffer (20 ml/1 g of powder) for 10 min in a Potter homogenizer and blended 2 hr with a magnetic stirrer, then centrifuged 20 min at 25,000 × *g;* the pellet was reextracted in the same way. Combined supernatants were drained through a column of DEAE-Sephadex A-50 equilibrated with the same buffer, and washed with 0.3 M Tris·HCl until absorbance of the effluent dropped to A_{280} = 0.05. Enzyme was then eluted with 0.3 M Tris buffer containing 2% $(NH_4)_2SO_4$, collecting eluate in 4 ml portions.

Adsorption on aluminum hydroxide C_γ. To the combined active fractions from the preceding step sufficient C_γ gel suspension was added for complete adsorption of the synthase. The gel was centrifuged, washed twice with 5% $(NH_4)_2SO_4$ solution in 0.3 M Tris buffer, and eluted with the same buffer containing 20% $(NH_4)_2SO_4$. In the eluate, the $(NH_4)_2SO_4$ concentration was raised to 0.7 saturation. After 2 hr, the mixture was centrifuged 20 min at 20,000 × *g;* the precipitate was dissolved in a minimal volume of 0.05 M Tris·HCl (pH 8.8).

Fractionation on Sephadex G-100, equilibrated with 0.05 M Tris buffer, was carried out in a column of 2.5 × 100 cm, into which the enzyme solution was allowed to drain, and then was eluted with the same buffer at a rate of 24 ml/hr.

Separation on hydroxyl apatite. Active fractions of the eluate were combined and treated with enough hydroxyl apatite suspension to completely adsorb the enzyme. After centrifugation (3000 × *g*) the supernatant was discarded, and the enzyme was brought into solution by extraction of the sediment with 0.3 M Tris buffer containing 8% of $(NH_4)_2SO_4$.

Preparative gel electrophoresis. The enzyme eluate thus obtained was concentrated to 3 ml on a Diaflo UM-3 ultrafilter; with addition of 0.05 M Tris buffer, ultrafiltration was repeated several times to reduce salt content. Three to five milliliters of the enzyme solution (5–10 mg of protein) were subjected to electrophoresis at 40 mA for 3 hr in an 0.5 × 9 × 9 cm slab of 7.5% polyacrylamide gel, as described by Davis (15). (CN)Ala synthase activity was then located and enzyme was eluted by blending the gel with 0.10 M Tris buffer.

Properties of the Enzyme. (CN)Ala synthase preparations thus purified are stable for more than a month when stored at −20°. Specific activity was 40–45 U/mg, i.e., it exceeded

200-fold that of the acetone powder extract, or about 4000-fold the specific activity of the seedling homogenate (Table 1). Disc-electrophoresis of the purified enzyme in polyacrylamide gel revealed the presence of one single, enzymatically active protein band.

Assays for contaminating pyridoxal-*P*-dependent activities (see refs. 18 and 19) demonstrated absence of γ-cystathionase (EC 4.4.1.1), cysteine lyase (EC 4.4.1.10), tryptophanase (EC 4.1.99.1), and serine sulfhydrase ≡ cystathionine β-synthase (EC 4.2.1.22).

As estimated by gel filtration through a column of Sephadex G-100, according to Andrews (20), (CN)Ala synthase has a molecular weight of approx. 52,000 (the molecular weight calculated from amino-acid composition determined in a BioCal analyzer was 51,600). Estimation of pyridoxal-*P* in the purest enzyme preparation by the method of Wada and Snell (21) showed a content of 0.84 mole of coenzyme per 1 mole of protein. The spectrum of the synthase is shown in Fig. 1. The results of isoelectrofocusing indicated a value of pH 4.7 for the enzyme's isoelectric point.

Substrate Specificity. From the data presented in Table 2, it is seen that, in addition to L-cysteine, β-chloro-DL-alanine can serve as an active amino substrate for (CN)Ala synthase. Under optimal conditions (at pH 8.8 in Tris buffer), the enzyme failed to use *O*-acetyl-L-serine as substrate; but it was shown that in this medium *O*-acetylserine is rapidly isomerized to *N*-acetylserine. The latter is not a substrate for (CN)Ala synthase (7). At less alkaline reaction (pH 7.5), and in increased concentration (10 mM), *O*-acetylserine was found to undergo slow β-replacement with [^{14}C]cyanide. With [^{14}C]-cyanide, it was shown that neither L-serine, nor L-alanine, *O*-phospho-L-serine, *S*-hydroxyethylcysteine, or (CN)Ala react as amino substrates (Table 2). The enzyme's action is nonreversible under the conditions used for the forward reaction.

The enzyme was observed to slowly utilize L-cystine in the reaction with cyanide. The reaction rate was less than 8% of the rate observed with L-cysteine (Table 2), the [^{14}C]cyanoalanine yield being greatly in excess of H_2S production. It is well known that organic disulfides, including cystine-containing peptides and proteins, readily undergo cleavage by nonenzymic cyanolysis to substituted thiols and thiocyanates, in alkaline media:

$$RS—SR' + HCN \rightarrow RSH + R'SCN.$$

On interaction of free cystine with cyanide, no accumulation of the expected β-thiocyanoalanine can be detected, owing to rapid cyclization of this compound to 2-aminothiazoline-4-carboxylic acid (22, 23). Our attempts to prepare (CNS)Ala by the procedure of Ressler *et al.* (24) for the synthesis of γ-thiocyano-α-aminobutyrate were unsuccessful.

It seemed likely that in the case of interaction of cystine with cyanide in the presence of (CN)Ala synthase, thiocyanoalanine produced by chemical cyanolysis might act as primary substrate in the enzyme-catalyzed β-replacement reaction:

$$(CNS)Ala + CN^- \rightarrow (CN)Ala + CNS^-.$$

Thus, the enzyme should produce 2 (CN)Ala + H_2S + CNS^- from cystine and cyanide; actually, thiocyanate ion produced in this reaction was estimated by photometry of its red Fe^{3+} salt (11). The optimal concentrations for the reaction were found to be 20 mM L-cystine and 50 mM KCN. In Fig. 2 the production of sulfide and CNS^- is plotted versus cystine concentration (with [KCN] = 50 mM).

TABLE 2. *Amino substrate specificity of (CN)Ala synthase**

Amino-acid substrate (2.5 mM)	Reaction rate (0 → 10 min), μmol/min: Cosubstrate: 2.5 mM KCN	Cosubstrate: 25 mM HS-EtOH
L-Cysteine	32.6	30.3
DL-(β-Cl)Ala	17.6	18.1
L-Cystine	2.6	—
LL-(CN)Ala	0	0
L-*S*-Hydroxyethyl-Cys	0	—
L-Ser; (*P*)Ser; L-Ala	0	—

* Products determined were: (^{14}CN)Ala, in column 2; (HOEtS)Ala, in column 3.

The suggested reaction mechanism is supported by the results of experiments where either iodoacetate or *p*-chloromercuribenzoate was incorporated into reaction mixtures with cystine and (CN)Ala synthase before KCN, to trap cysteine produced by cyanolysis and prevent its enzymic β-replacement reaction. Under such conditions, the yield of CNS^- remained almost unchanged, while H_2S formation was markedly depressed.

Investigation of co-substrate specificity of the synthase (Table 3) showed that 2-mercaptoethanol (K_m = 20 mM) can be utilized instead of KCN (K_m = 0.55 mM); with either cysteine or (Cl)Ala as the amino substrate, *S*-hydroxyethylcysteine was identified as end-product. Some other small thiol compounds, e.g., methane- and ethanethiol, cysteamine, can likewise serve as cosubstrates, but the enzyme failed to utilize butanethiol, benzylmercaptan, thioglycolate, 3-mercaptopropionate, dithiothreitol, thiocyanate, or sulfite (Table 3).

Inhibitor Sensitivity. The effects on (CN)Ala synthase of some representative compounds inhibitory to other pyridoxal enzymes are summarized in Table 4. The synthase is highly sensitive to carbonyl reagents, in particular those structurally

TABLE 3. *Cosubstrate specificity of (CN)Ala synthase**

Cosubstrate	Concentration, mM	Reaction rates, % of rate with cyanide
KCN	2.5	100
Methanethiol	~25†	>100
Ethanethiol	25	45
n-Butanethiol	25	6
t-Butyl and benzylmercaptan; thioglycolate, 3-mercaptopropionate	25	0
2-Mercaptoethanol	25	83
Cysteamine	25	23
L-Homocysteine; reduced glutathione; dithiothreitol; Na_2SO_3; KCNS	2.5	0

* Initial rate of H_2S formation from L-cysteine, in percent of rate in standard assay with KCN.

† Concentration inexact, owing to high volatility of the compound.

TABLE 4. *Inhibition of (CN)Ala synthase**

Inhibitors	Concentrations (M)			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Aminooxyacetic acid	100	93	74	30
3-Aminooxypropionic acid	100	72	37	10
DL-Cycloserine	0	—	—	—
D-Penicillamine	0	—	—	—

* Percent inhibition compared with control sample (cysteine + cyanide). Inhibitors were preincubated with enzyme for 5 min at room temperature.

analogous to substrates, e.g., aminooxyacetate, 3-aminooxypropionate. Like other pyridoxal-*P*-dependent enzymes, it is inactivated by $NaBH_4$. However, (CN)Ala synthase proved remarkably refractory to some potent active-site directed inhibitors of many pyridoxal enzymes, such as DL-cycloserine and D-penicillamine, in concentrations up to 10 mM (Table 4; see *Discussion*).

Protium–Tritium Exchange in Amino Substrate and Product. L-[β-^{3}H]Cysteine, diluted with nonlabeled cysteine to specific radioactivity of 6.7×10^4 cpm/μmol, was used to test for eventual dissociation of H atoms in β-position in the reaction catalyzed by (CN)Ala synthase. Upon addition of KCN and enzyme, the samples were incubated at 30°; then solvent water was distilled off, and aliquots of distillate were used for estimation of labeled β-hydrogen released into the medium. After 1 or 2 hr incubation, radioactivity of the distillate did not exceed the background count. This testifies to the absence of enzyme-catalyzed release of β-H from cysteine.

Next, the exchange of α-H in substrate molecules was studied on interaction with (CN)Ala synthase. In Fig. 3 the time-course is shown of tritium incorporation from ^3H_2O-containing medium into reaction product and substrate (L-cysteine), on incubation with (CN)Ala synthase in presence of cosubstrate (HS-EtOH or KCN) and in its absence. Incorporation of ^{3}H into products was measured by estimation of the quantity and specific radioactivity of amino acids recovered from paper chromatograms of aliquots taken from the samples at specified time intervals. Relative specific radioactivity (r.s.r.) of (CN)Ala (α-H) produced was 100% irrespective of the duration of incubation (Fig. 3, curve 4). The relatively slow rate of ^{3}H incorporation into nontransformed cysteine, catalyzed by (CN)Ala synthase in absence of cosubstrate (curve 1), was markedly increased in the presence of added cosubstrate, namely, 2-mercaptoethanol (curve 2) or, in particular, KCN; in the latter case (curve 3), r.s.r. calculated for one H atom attained 85% after 2 hr. It is thus clear that in L-cysteine only one hydrogen atom (the α-H) is exchanged on interaction with (CN)Ala synthase, the exchange being slow without cosubstrate and fairly rapid in its presence, in accordance with the reaction mechanism suggested (25) for β-replacement-specific pyridoxal-*P*-dependent lyases (see below).

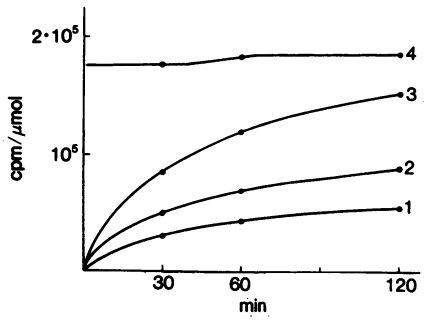

FIG. 3. Time course of α-H exchange in substrate (L-cysteine, curves 1–3) and product (cyanoalanine, curve 4) catalyzed by (CN)Ala synthase, without cosubstrate (curve 1), with 2-mercaptoethanol (curve 2) or KCN (curves 3 and 4). The ordinate is specific radioactivities in cpm/μmol (specific radioactivity of solvent $^3H_2O = 1.8 \times 10^5$).

DISCUSSION

Evidence reported earlier (8, 9, 25) demonstrated that some pyridoxal-*P*-dependent enzymes playing important roles in the metabolism of hydroxy and sulfur amino acids, such as cysteine lyase, serine sulfhydrase, or cystathionine β-synthase, belong to a special subgroup of lyases which catalyze exclusively reactions of β-replacement by a mechanism differing substantially from that of α,β-elimination. A common feature of these lyases is the utilization of certain thiols as cosubstrates, e.g., of 2-mercaptoethanol (9, 18, 19). The main biological function of (CN)Ala synthase is evidently β-replacement of HS in cysteine by CN, but the enzyme from blue lupine was shown to catalyze also, at a comparable rate, the reaction of cysteine with mercaptoethanol.

We prepared the enzyme from acetone powder of the mitochondria of blue lupine seedlings, made as described by Hendrickson and Conn (7); further purification by their method was not quite satisfactory, in our hands. Effective steps in the modified procedure here reported were the fractionation of extracted protein on DEAE-Sephadex and, in particular, adsorption of the enzyme on Cγ gel and on hydroxylapatite (Table 1). By means of this procedure, (CN)Ala synthase preparations were obtained of improved specific activity (40–45 U/mg) and in recoveries (25%) more than twice as high as reported in ref. 7.

Activity ratio of the enzyme preparation in (CN)Ala synthesis and in *S*-hydroxyethylcysteine formation was nearly constant—approximately 1.0 on all levels of purification. This shows that (CN)Ala synthase is able, like the other β-replacing lyases, to use HS-EtOH as cosubstrate; it also uses some other small aliphatic thiols, but differs from serine sulfhydrase (and cysteine lyase) in failing to utilize homocysteine (and sulfite, respectively) as cosubstrates (Table 3). (Cl)Ala is an adequate primary substrate for (CN)Ala synthase, as it is for serine sulfhydrase (9), but in contrast to the latter enzyme, (CN)Ala synthase does not utilize either L-serine (and most of its analogs) or L-cysteine thioethers as amino-acid substrates (Table 2).

A noteworthy finding was the enzyme's ability to catalyze interaction of L-cystine, at a fairly slow rate, with cyanide (Table 2), producing thiocyanate in addition to sulfide and (CN)Ala (see Fig. 2). The phenomenon is similar to one observed by Ressler *et al.* (24), who described a pyridoxal enzyme from *Chromobacterium violaceum* which catalyzes formation of γ-cyano-α-aminobutyrate and CNS^- from homocystine (or synthetic γ-thiocyano-α-aminobutyrate) and KCN. In the present case, enzymic β-replacement is evidently preceded by nonenzymic interaction of cyanide with cystine (cyanolysis), producing L-cysteine and (CNS)Ala. The latter, unstable compound is rapidly cyclized to 2-aminothiazoline-

4-carboxylic acid (22, 23) in the absence of (CN)Ala synthase. In the presence of this enzyme and of cyanide, (CNS)Ala undergoes enzymic β-replacement, yielding (CN)Ala + CNS^-. Consistent with the inference that (CNS)Ala can serve as a primary substrate for (CN)Ala synthase, is the finding that the yield of CNS^- remains practically unchanged, while sulfide production is suppressed, on incorporation of a thiol-binding reagent (e.g., *p*-chloromercuribenzoate) into the reaction mixture, so as to trap the cysteine produced by cyanolysis of cystine.

Evidence casting light on the mode of action of (CN)Ala synthase was derived from investigation of its inhibitor susceptibility, on one hand, and of the protium–tritium exchange it catalyzes, on the other.

The enzyme's most potent inhibitors are carbonyl reagents blocking the formyl group of pyridoxal-*P*, in particular, aminooxyacetate and 3-aminopropionate (Table 4).

Especially important (and unrelated) peculiarities of (CN)Ala synthase are revealed by its complete insensitivity to inhibition by DL-cycloserine and by penicillamine in high concentrations (10 mM). Resistance to inhibitors of these classes is common to the β-replacing lyases (8, 9, 25).

High sensitivity to inactivation by DL-cycloserine and related aminoisoxazolidones is typical of those pyridoxal enzymes whose catalytic mechanism embodies obligatory pyridoxamine-*P* ketimine intermediates (aminotransferases, aspartate β-decarboxylase, γ-specific lyases, etc.); these agents do not act on enzymes forming no ketimine intermediates, e.g., on amino-acid α-decarboxylases (8). Braunstein's suggestion (for details, see ref. 8) that resistance of a pyridoxal enzyme to inactivation by aminoisoxazolidones can serve as indication of a reaction mechanism requiring no ketimine intermediate is supported by the finding that, as expected, (CN)Ala synthase shares with the other β-replacing lyases (compare refs. 8, 9, and 25) this resistance to cycloserine and its analogs.

Penicillamine and other aminothiols (especially those structurally analogous to amino substrates) are known to inhibit efficiently a number of pyridoxal enzymes by condensing with pyridoxal-*P* in the active site to form stable thiazolidine derivatives. On grounds of the presumable stereochemistry of substrate binding in the active centers, it was predicted that lyases exclusively catalyzing β-replacement should be refractory to inhibition by aminothiols, in contrast to the highly susceptible eliminating and ambivalent β- and γ-lyases (for discussion, see refs. 8 and 25). This prediction was confirmed for three β-replacing lyases in the previous papers just cited. The data reported above, showing that (CN)Ala synthase is similarly insensitive to penicillamine (Table 4), lend support to its allocation in the subfamily of β-replacement-specific lyases.

The features of protium–tritium exchange catalyzed by (CN)Ala synthase (see *Results* and Fig. 3) are fairly similar to those recently reported by Tolosa *et al.* (25) for serine sulfhydrase and cysteine lyase. With these two β-replacing lyases, in marked contrast to the α,β-eliminating or plurifunctional ones (e.g., tryptophanase, γ-cystathionase), it was found that isotopic H exchange (*1*) requires the presence of adequate cosubstrate, (*2*) mostly is not catalyzed in substrate analogs that do not undergo the complete reaction, (*3*) is limited to the α-H atom, (*4*) its rate is of the same order as that of the overall replacement reaction.

From these and other properties of the selectively β-replacing lyases, it was inferred that they do not comply to the general reaction scheme accepted by Snell and other American authors (26) for pyridoxal-*P*-dependent lyases (see ref. 25, scheme 1), involving enzyme-bound α,β-unsaturated coenzyme-substrate Schiff-base intermediates both in the case of β-replacement and of elimination reactions.

The failure of β-replacement-specific lyases, including (CN)-Ala synthase, to effect α,β-elimination reactions, and the other known features of these enzymes render formation of α,β-unsaturated pyridoxylidine aldimines as real reaction intermediates in their catalytic mechanism very unlikely; see the alternative reaction mechanism for β-replacement outlined by Braunstein and Shemyakin (ref. 25, scheme 2) and the discussion of the molecular catalytic mechanisms of α,β-eliminating and β-replacing lyases in that paper and in refs. 8 and 9.

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