

Purification and Properties of γ -Alanine Synthase from Calf Liver

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Abstract: γ -Alanine synthase (EC 3.5.1.6) catalyzes the conversion of *N*-carbamyl- γ -alanine to γ -alanine, ammonia and CO₂. The enzyme has been purified to apparent homogeneity from calf liver. The molecular size, pH optimum and substrate specificity have been determined. Sequence alignment of γ -alanine synthases with *N*-carbamyl-D-amino acid amidohydrolase from *Agrobacter sp.* revealed the conservation of a catalytically important triad Glu-Lys-Cys, most likely involved in the breakdown of *N*-carbamyl- γ -alanine.

Keywords: alanine, alanine synthases, pyrimidine degradation, neurotransmitter, *N*-carbamyl- γ -alanine, *N*-carbamyl- γ -aminoisobutyrate.

Abbreviations: AS, γ -alanine synthase; PTC, phenyl isothiocyanate; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; DTE, dithioerythritol; HPLC, high-pressure liquid chromatography; DCASE, *N*-carbamyl-D-amino acid amidohydrolase; CSHase, *N*-carbamyl sarcosine amidohydrolase DHU, dihydrouracil; DHT, dihydrothymine; NCBA, *N*-carbamyl-DL- γ -alanine; NCIB, *N*-carbamyl-DL- γ -aminoisobutyrate; 4UBA, 4-ureidobutyrate; PA, propionate; GAMA, glutarate monoamide; TCA, trichloroacetic acid.

INTRODUCTION

γ -Alanine synthase (AS) (EC 3.5.1.6) catalyzes the third and final step of the reductive pyrimidine catabolic pathway [1]. The enzyme is also known as *N*-carbamoyl- γ -alanine amidohydrolase or γ -ureidopropionase. In the reaction catalyzed by AS, *N*-carbamoyl- γ -alanine or *N*-carbamoyl- γ -aminoisobutyrate is irreversibly hydrolyzed to CO₂, NH₃ and γ -alanine or γ -aminoisobutyrate, respectively.

Degradation of uracil is the only pathway to provide γ -alanine in mammals [2]. Microorganisms can additionally form γ -alanine either by direct γ -decarboxylation of L-aspartate [3] or by degradation of polyamines [4]. γ -Alanine is a precursor of pantothenate and coenzyme A [5], and also plays a role in pigmentation of insect cuticle [6] and in the fungal cell wall [7]. Due to its structural similarity to GABA (an excitatory transmitter in addition to its well-known inhibitory function [9]), γ -alanine is thought to function as a neurotransmitter [8]. Disorders in γ -alanine metabolism in humans, such as hyper- γ -alaninemia are associated with severe neural dysfunction, seizures and death [10, 11]. Recently, the first patient with a putative defect in γ -alanine synthase has been reported [12]. A 17-month-old girl presented with muscular hypotonia, dystonic movements, scoliosis, microcephaly and severe developmental delay. She had normal concentrations of thymine and uracil, while the concentrations of dihydropyrimidines were moderately elevated. In contrast, *N*-carbamyl- γ -alanine and *N*-carbamyl- γ -aminoisobutyrate were strongly increased [13]. It is thus important to understand the mechanism of the AS.

Eukaryotic γ -alanine synthases have been purified from *Euglena gracilis* [14], mouse [15], and rat liver [16]. Recently, AS was also isolated and purified from *Saccharomyces kluveri* [17, 18]. A cDNA encoding the

AS gene product has been cloned and sequenced from rat [19], human [20] and the yeast *Saccharomyces kluveri* [21]. In addition, a *S. kluveri pyd3* mutant that is unable to grow on *N*-carbamyl- γ -alanine as the sole nitrogen source and exhibits diminished γ -alanine synthase activity was used to clone analogous genes from other eukaryotes. Putative *PYD3* sequences from *Saccharomyces kluveri* (*Sk*), *Dictyostelium discoideum* (*Dd*), and *Drosophila melanogaster* (*Dm*) complemented the *pyd3* defect [17]. Based on a comparison of primary sequence and phylogenetic analysis of ASs these enzymes can be assigned to one of three subfamilies [17]. The majority of the eukaryotic ASs fall into one subfamily, including the *Dd* and *Dm PYD3* gene products. In contrast, the AS from *Saccharomyces kluveri* (*Sk AS*) shares only a limited sequence similarity with the eukaryotic ASs, but instead is closely related to bacterial *N*-carbamyl-L-amino acid amidohydrolases, and is thus a member of the second subfamily. *PYD3* expression in yeast appears to be induced by dihydrouracil (DHU) and *N*-carbamyl- γ -alanine (NC A), but not by uracil [17]. A third subfamily of enzymes includes bacterial and archaeobacterial *N*-carbamyl-D-amino acid amidohydrolases.

Rat AS exists as an inactive trimer, a stable active homohexamer and active homododecamer, depending on the presence or absence of allosteric effectors [22]. A model suggesting an allosteric regulatory site distinct from the catalytic site has been proposed [23].

In this study we report the purification and physical and kinetic characterization of a γ -alanine synthase from calf liver.

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MATERIALS AND METHODS

Chemicals

Phenyl isothiocyanate and triethylamine were from Pierce Chemical Company (Rockford, Ill.). N-carbamyl- α -alanine, α -alanine, catalase, dithioerythritol, and propionic acid were obtained from Sigma (St. Louis). N-carbamyl-DL- α -aminoisobutyric acid was prepared according to a published method [24]. To remove free α -alanine, 1 g NCBA and 3 g NaHCO₃ were dissolved in 30 mL water. After addition of 2 mL benzoyl chloride the solution was maintained at 46°C for 2 hr and then the pH was adjusted to 4.3 with 6 N HCl. The precipitated benzoyl- α -alanine is removed by centrifugation and benzoic acid in the supernatant extracted 3-times with diethyl ether. The NCBA treated as above contains less than 0.01 % free α -alanine. All other chemicals were of the highest purity commercially available.

Purification of Calf Liver AS

Calf liver acetone powders (100 g) were suspended in 400 mL 35 mM potassium phosphate buffer containing 10 mM propionate and 3 mM DTE, stirred for 30 min and finally centrifuged (20 min at 27,500 x g). The crude extract was then heated in a metal beaker for 7 min at 50°C, quickly cooled to 10°C and again centrifuged (10 min 48,000 x g). The supernatant from the heat treatment step was subjected to a 30-52.5 % ammonium sulfate fractionation. The pellet was dialyzed against 1.3 M potassium phosphate, pH 7.0 containing 1 mM DTE followed by chromatography on an octyl-Sepharose column (2.4 x 35 cm). After washing with 5 column volumes of 1.3 M phosphate, pH 7.0 a linear reverse gradient 1.3 M to 0.1 M phosphate was used at a flow rate of 40 mL/h to develop the chromatogram. The

AS-active fractions were concentrated using an Amicon ultrafiltration device with a PM-10 membrane, and the concentrate was then dialyzed against 10 mM potassium phosphate, pH 7.0, applied to a DEAE-Sepharose FF column (1.8 x 35 cm), and eluted with a 0-0.15 M KCl gradient in 10 mM potassium phosphate, pH 7.0. The active fractions were pooled, concentrated, and dialyzed against 10 mM potassium phosphate, pH 6.0. Chromatography on a CM-cellulose column (1.6 x 12 cm) and elution with a pH gradient (pH 6.0 to 6.7) (2 x 1000 mL) resulted in pure calf liver AS with an overall yield of 46 % and an overall purification of about 1000-fold (see Table 1).

Enzyme Assay

The α -alanine content of an assay mixture was determined quantitatively by derivatization with phenyl isothiocyanate followed by reversed-phase HPLC chromatography [25]. α -Alanine synthase was preincubated in 10 mM potassium phosphate, pH 7.0 at 37°C. After 3 min the reaction was initiated by addition of NCBA. After a 5 min reaction in a closed reaction tube the enzymatic reaction was stopped by heat treatment (5 min, 95°C). (The time course of the reaction indicates it is linear for more than 5 min. under all conditions.) The sample was stored in ice for 15 min and then centrifuged at 20,000 g for 10 min, to remove denatured protein. An aliquot (0.5 mL) of the supernatant was dried in a Speed-Vac and the pellet was

dissolved in 20 μ L ethanol, water, triethylamine (2:2:1 (v,v)) and again evaporated. The pellet was then mixed with 20 μ L of coupling solution (ethanol, water, triethylamine, PITC, 7:1:1:1 (v/v)) and reacted for 30 min at room temp. After evaporation, PTC- α -alanine was dissolved in 50 mM acetate, pH 6.5 plus 15% acetonitrile (buffer A). An aliquot was applied to a reverse-phase HPLC column equilibrated with the buffer A at room temperature. Isocratic elution with buffer A at a flow rate of 1 mL/min and detection at 245 nm reveals pure PTC- α -alanine. A calibration curve was established using different amounts of PTC- α -alanine.

Molecular Mass Determination

The subunit molecular mass of the calf liver α -alanine synthase was estimated by SDS/PAGE in a discontinuous buffer system [26]. The mass of the native protein was determined by native gel electrophoresis on 4-20% gradient gels (Bio-Rad) in Tris-glycine, pH 8.5) in a GibcoBRL Mini-V 8 x 10 vertical gel electrophoresis system (Life Technologies, Inc.). Gels were stained for protein with Coomassie Blue or silver stain [27]. Urease (trimer 272 kDa, dimer 181 kDa, monomer 91 kDa), chicken egg albumin (43 kDa), bovine serum albumin (67 kDa) and carbonic anhydrase (30 kDa) were used as protein standards in native gels, while phosphorylase b (94 kDa), bovine serum albumin, ovalbumin and carbonic anhydrase were standards for SDS-PAGE. In addition, the molar size of native AS was estimated by chromatography on Sephacryl S-400 HR (1.6 x 92.5 cm). The column was equilibrated with 50 mM potassium phosphate, pH 7.0, containing 150 mM KCl and 1 mM DTE. The proteins were eluted with the equilibration buffer at a flow rate of 20 mL/h. The column was calibrated with urease (480 kDa), pyruvate kinase (237 kDa), aldolase (158 kDa) and bovine serum albumin (67 kDa) as protein standards.

Kinetic Data Processing

Data were fitted using the appropriate rate equations and the Fortran programs developed by Cleland [28]. Reciprocal initial velocities were plotted versus reciprocal substrate concentrations and all plots were linear. Data for NBCA saturation curves were fitted using eq. 1.

$$v = \frac{VA}{K_a + A} \quad [1]$$

Data for linear competitive and noncompetitive inhibition were fitted using eq. 2 and 3, respectively.

$$v = \frac{VA}{K_a \left(1 + \frac{I}{K_{is}}\right) + A} \quad [2]$$

$$v = \frac{VA}{K_a \left(1 + \frac{I}{K_{is}}\right) + A \left(1 + \frac{I}{K_{ii}}\right)} \quad [3]$$

In equations 1-3, V is the maximum velocity; A and I are concentrations of reactant and inhibitor, respectively; K_a is

Table 1. Purification of α -Alanine Synthase.

Procedure	Volume mL	Protein mg	Activity U ^a	S. A. U/mg	Yield %
Acetone powder	570	9400	9690	1.0	100
Heat treatment	515	8240	9600	1.9	99
(NH ₄) ₂ SO ₄ 32-52.5%	23.5	2150	8800	4.1	91
Octyl Sepharose	60.5	385	7650	19.9	79
DEAE Sepharose	14.5	121	7320	60.5	76
CM cellulose	10.2	4.4	4458	1013.2	46

^aU, 1 nmole of NCBA hydrolyzed per min per mg protein; S.A., specific activity.

Table 2. Molecular Size of α -Alanine Synthases.

	Rat Liver [23]	<i>Homo sapiens</i> [20]	<i>S. kluyveri</i> [18]	Calf Liver
Native Molecular Mass kDa	240	80	113	240
Subunit Molecular Mass kDa	42	43	40	40
Number of Subunits	6	2	2	6
Substrate Specificity	NCBA	NCBA	NCBA	NCBA

the Michaelis constant for reactant; K_{is} and K_{ii} are slope and intercept inhibition constants, respectively.

Protein Determinations

Protein concentrations were determined by the method of Bradford [29] using bovine serum albumin as standard.

RESULTS

Sequence Alignment

The enzymes of the pyrimidine degradative pathway have thus far been studied only in mammals and yeast. The sequence alignments of the various α -alanine synthases suggest that there is 20-30 % identity among the eukaryotic α -alanine synthases as well as the sequences of *Arabidopsis thaliana*, *Drosophila melanogaster* and *Dyctyostelium discoideum* but not for *Saccharomyces kluyveri* [17].

Purification of Native Calf Liver α AS

Purification of the calf liver α AS was accomplished from an acetone powder of a crude extract by heat treatment (7 min, 50°C), ammonium sulfate fractionation (30 to 52.5 % saturation), chromatography on octyl-Sepharose (pH 7.0), DEAE-Sepharose FF (pH 7.0) and CM-cellulose (pH 6.0). Homogeneous protein was obtained in 46 % yield with an overall purification of about 1,000-fold (Table 1) and a specific activity of 1,013 nmole NCBA hydrolyzed per min and mg protein.

Molecular size of Native Enzyme and of Its Subunits

The molecular mass of native calf liver α AS is 221 kDa and 240 \pm 4 kDa as determined by molecular sieve

chromatography on Sephacryl S-400 HR and polyacrylamide gradient gel electrophoresis, respectively. The molecular mass of the subunit is 40 \pm 5 kDa according to SDS-PAGE, consistent with a homohexameric enzyme. All data are summarized in Table 2.

Kinetic Measurements

Optimal enzymatic activity for calf liver α AS is observed over the pH range 5.7 to 7.0. The K_m values of NCBA and N-carbamyl-DL- α -aminoisobutyrate for the calf liver α AS are 21.7 and 8.4 μ M, respectively. The maximum velocity for calf liver α AS is 1.1 and 0.7 μ moles/min/mg protein using NCBA and N-carbamyl-DL- α -isobutyrate as substrates, respectively (see Table 3). The K_m value for the rat enzyme is 2-fold lower than that of the calf liver enzyme. It is questionable whether this is due to the fact that the purification procedure of the calf liver enzyme includes a heat step, which is claimed by the Traut group to destabilize the protein and increase the K_m value significantly. Much higher values of the K_m have been observed for the rat enzyme [16].

Inhibitors have been identified for the calf liver enzyme. The α AS from calf liver is competitively inhibited by 4-ureidobutyrate, propionate and glutarate monoamide with K_i values of 1.6, 240 and 630 μ M, respectively, at 37°C and pH 7.0 (see Table 3). GAMA is a much better competitive inhibitor for dihydropyrimidinase than for calf liver α AS [30].

DISCUSSION

In mammalian systems, and some bacteria, uracil and thymine are degraded in a three-step catabolic pathway using the enzymes dihydropyrimidine dehydrogenase, dihydro-

Table 3. Kinetic Parameters of α -Alanine Synthase.

	Calf Liver	Rat Liver [23]
K_{NCBA} (μM)	21.7	8.0
V_{NCBA} (nmol/min mg)	1,113	877
V/K_{NCBA} ($\text{min}^{-1}\text{mg}^{-1}$)	51.3	109.6
K_{NCIB} (μM)	8.4	nd
V_{NCIB} (nmol/min mg)	710	nd
V/K_{NCIB} ($\text{min}^{-1}\text{mg}^{-1}$)	84.5	nd
$K_{i\ 4\text{-UBA}}$ (μM)	1.6	nd
$K_{i\ \alpha\text{-Ala}}$ (μM)	nd	1.08
$K_{i\ \text{PR}}$ (μM)	240	90
$K_{i\ \text{GAMA}}$ (μM)	630	nd

pyrimidine amidohydrolase and α -alanine synthase. The α -alanine synthase has thus far only been purified and characterized in rat liver [22, 23, 31], although a preliminary report has been published on the calf liver enzyme [32]. The *Sk* AS was recently cloned, overexpressed and purified but not fully characterized [17].

Sequences of AS from different sources, when aligned show a remarkable similarity. It is proposed that the ASs belong to the amidohydrolase superfamily [33] containing proteins that catalyze various hydrolytic reactions at carbon and phosphorus centers. The superfamily can be divided into three subsets of enzymes, on the basis of three-dimensional structure of amidohydrolases that vary in the presence of metal ions in the active site. Family I includes enzymes with binuclear metal centers, such as dihydroorotase [34], phosphotriesterase [35] hydantoinase [36] and urease [37]. Family II contains proteins with a mononuclear metal center like adenosine deaminase [38], while the third family includes proteins that accomplish hydrolysis by a nucleophilic mechanism as suggested for N-carbamyl-D-amino acid amidohydrolase from *Agrobacterium. sp.* [39].

Very recently the three-dimensional structure of α -alanine synthase from *Saccharomyces kluveri* was determined at a resolution of 2.7 Å [18]. It shares a structural scaffold with dinuclear zinc dependent exopeptidases, which are very different from the structures of dihydropyrimidinases, hydantoinases and dihydroorotases [34, 36, and 40]. The subunit of the homodimeric *Sk* AS consists of two domains, a large catalytic domain with a di-zinc metal center, which represents the active site, and a smaller domain mediating the majority of the inter-subunit contacts. Both domains exhibit a mixed β/α topology. Only two other enzymes that catalyze the hydrolysis of N-carbamyl moieties, N-carbamyl-D-amino acid amidohydrolase from *Agrobact. sp.* (DCase) [39] and N-carbamyl sarcosine amidohydrolase (CSHase) [41], have a three dimensional structure determined. But neither of these enzymes has a divalent metal ion bound to its active site. In contrast, the active site residues of DCase (E46, K126 and C171) have a geometry similar to those of the homologous residues (D51, K144 and C177) in CSHase, suggesting that a putative catalytic triad, Glu(Asp)-Lys-Cys, is essential for some N-carbamylamide-hydrolyzing functions. Interestingly,

these catalytically important amino acid residues are completely conserved in α -alanine synthase genes of human and rat liver, as well as in *Drosophila melanogaster* and *Arabidopsis thaliana*, suggesting a similar structure of their active sites. The corresponding critical amino acids in human α -alanine synthase are E119, K192 and C229. Based on the structure of the DCase [36], a similar nucleophilic chemical mechanism for eukaryotic ASs can be proposed in which the cysteine thiol acts as a nucleophile and the Glu/Lys act as acid-base catalysts.

The calf liver AS is a hexamer like the corresponding rat liver enzyme with a subunit molecular size of about 40 kDa. The hexameric structure of the calf liver enzyme does not change in the presence of NCBA or α -alanine, in contrast to the rat liver enzyme [23].

The kinetic properties of calf liver AS are very similar to those of the rat liver enzyme with the exception of the allosteric behavior found for the rat liver enzyme, which is not seen for the calf liver AS, i.e. neither α -alanine nor N-carbamoyl- α -alanine changes the molecular size of the native enzyme. It seems unlikely that the allosteric properties have been lost due to the heat treatment applied to the calf liver AS in the presence of the competitive inhibitor propionate, since the K_m values for NCBA and the K_i value for propionate are only different by a factor of 2-3. (A significant difference in K_m and K_i values of α -alanine synthase was proposed to be due to destabilization of the protein [19].) NCIB, which has an additional methylene compared to NCBA, has a 5-fold lower K_m but a V_{max} that is 60% compared to NCBA, the natural substrate, (Table 3). As a result, the V/K values, 84 and 51 min^{-1} , are very similar for the two substrates, suggesting the bulkier isobutyrate side chain has little effect on catalytic efficiency.

The tightest binding inhibitor for calf liver AS is 4-ureidobutyrate, a substrate analog harboring an additional methylene group between the ureido and the carboxylate groups, suggesting a longer more extended molecule has a better active site fit, but is not properly positioned to act as a substrate. As expected, elimination of the α -ureido group, propionate, gives a significant decrease in affinity, > 100-fold. The affinity of α -alanine should be very similar for the enzymes from calf and rat livers given the data in Table 3, and thus the addition of a β -amino group significantly increases the affinity of propionate, suggesting the presence of an active site group that interacts with the β -amino group. This result is not surprising since the β -amino group must be protonated in the course of the reaction. In agreement with this suggestion, the affinity of enzyme for GAMA, which has an alkyl side chain identical to that of 4-UBA, but is missing a nitrogen at the 4-position, has an approximately 400-fold decreased affinity compared to 4-UBA. Thus, data suggest minimally that 1) an active site group is required to bind the carboxylate, likely to orient reactant for catalysis, and 2) an active site group is required to act as a general acid to protonate the β -amino group as it is formed.

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