Vol. 185, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1992 Pages 60-68

THE ENANTIOSELECTIVE PARTICIPATION OF (S)- AND (R)-DIAMINOVALERIC ACIDS IN THE FORMATION OF δ-AMINOLEVULINIC ACID IN CYANOBACTERIA

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Received April 2, 1992

Although it is recognized that 4,5-diaminovaleric acid, formed from glutamate 1-semialdehyde, functions as the intermediate in the last step of δ-aminolevulinic acid formation from glutamate, the enantioselectivity of the participating glutamate 1-semialdehyde aminotransferase for 4,5-diaminovaleric acid has remained unknown. In the present work the involvement of (S)- and (R)-4,5-diaminovaleric acids, newly available by organic synthesis, was investigated, using glutamate 1-semialdehyde aminotransferase from *Synechococcus*. The preferred enantiomer was (S)-4,5-diaminovalerate. In experiments on the transformation of (S)-4,5-diaminovalerate to δ-aminolevulinate it was found that glutamate 1-semialdehyde aminotransferase was unusual among aminotransferases in that the common amino acceptors pyruvate, oxaloacetate, α-ketoglutarate were inactive, while 4,5-dioxovaleric acid could be utilized as a sluggish amino acceptor in place of glutamate 1-semialdehyde. In conclusion, glutamate 1-semialdehyde aminotransferase is highly but not absolutely enantioselective for (S)-4,5-diaminovaleric acid, and 4,5-dioxovaleric acid can function as amino acceptor not because of a physiological role in the C_5 pathway of δ-aminolevulinic acid formation, but because of its structural resemblance to glutamate 1-semialdehyde.

Glutamate 1-semialdehyde (GSA) aminotransferase, the enzyme that transforms GSA into δ-aminolevulinate (ALA) (1) is unusual: unlike other aminotransferases it catalyzes a two-step reaction sequence without the participation of an externally added amino acceptor or donor. This economical condition is the result of the circumstance that GSA is distinguished from other aminotransferase substrates in carrying both the amino group and the oxygen function that participate in the transamination. Thus the product of the first transamination becomes the substrate for the second transamination (Scheme 1). Since the enzyme catalyzes the shift of an amino group, it has been classified as a mutase (EC 5.4.3.8). However, the type of chemical change brought about by

Abbreviations: ALA, δ-aminolevulinate; GSA, glutamate 1-semialdehyde.

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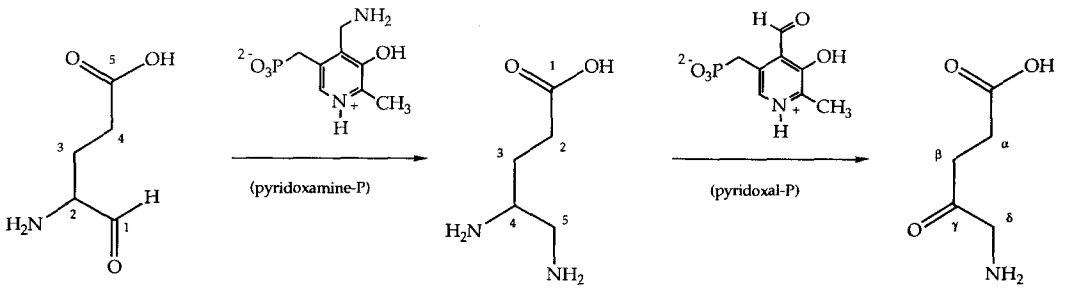

Glutamate 1-Semialdehyde (GSA) 4,5-Diaminovaleric acid (DAVA) δ-Aminolevulinic acid (ALA)

Scheme 1

the enzyme raises the mechanistic expectations of an aminotransferase, since the net result, unlike that for other mutases, is a reciprocal replacement of oxygen and amino functions. The tentative suggestion of an intramolecular transamination (1) was ruled out on the basis of NMR evidence (2). The absence of an intramolecular transamination did not, however, directly suggest Scheme 1 as the reaction sequence from GSA to ALA. Thus for the purified dimeric enzyme from barley, which, in distinction to the enzyme from peas (3), appears not to contain vitamin B_6-type cofactors (4,5), it was suggested that two molecules of GSA interact directly (6), with the intermediate formation of a double Schiff base (7,8). In contrast, for GSA aminotransferases that do contain participating vitamin B_6-type cofactors, such as the monomeric GSA aminotransferase from the cyanobacterium *Synechococcus* (4), two contrasting transamination sequences could be envisaged (9) to lead from GSA to ALA: as the first step one could postulate either the gain by GSA of an amino group from pyridoxamine-*P*, resulting in the formation of the ornithine isomer, 4,5-diaminovaleric acid and pyridoxal-*P* (Scheme 1, and Fig. 1A), or the loss by GSA of its amino group to pyridoxal-*P*, leading to 4,5-dioxovaleric acid, an oxidized form of α-ketoglutarate, and pyridoxamine-*P* (Fig. 1B). In the first alternative the other amino group of the intermediate diamine would now be lost to pyridoxal-*P*, forming ALA and regenerating pyridoxamine-*P*, while in the second alternative the aldehyde carbon of the intermediate dioxo compound would now gain an amino group from pyridoxamine-*P*, with the formation of ALA and the regeneration of pyridoxal-*P*. Various considerations had pointed to the first of these two schemes (4,8,9,10), but only recent kinetic (11) and spectroscopic (12) evidence, obtained with the *Synechococcus* GSA aminotransferase, firmly established the occurrence of this alternative: 4,5-diaminovalerate participates and 4,5-dioxovalerate does not. It was found, in fact (11,12), that small amounts of the added diamine increase the rate of conversion of GSA to ALA, almost certainly by accelerating the rate of reconversion of pyridoxal-*P* to pyridoxamine-*P*, while the added dioxo compound was strongly inhibitory.

It is clear, therefore, that 4,5-diaminovalerate is an intermediate that, in addition to glutamyl-$tRNA^{Glu}$ and GSA, participates in the conversion of glutamate to

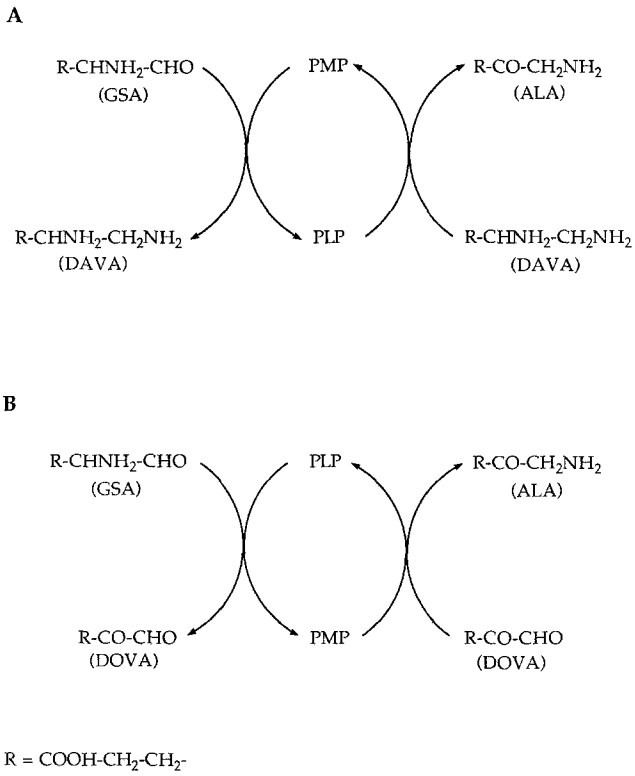

Fig. 1. Two alternative potential modes of δ-aminolevulinate (ALA) formation from glutamate 1-semialdehyde (GSA). **A**: 4,5-diaminovalerate (DAVA) is formed by transamination at the terminal carbon, and loses the amino group at the proximal carbon to yield ALA. **B**: 4,5-dioxovalerate (DOVA) is formed by transamination at the proximal carbon and gains the amino group at the terminal carbon to yield ALA. PMP and PLP represent the enzyme-bound forms of pyridoxamine-*P* and pyridoxal-*P*.

ALA. The experiments reported thus far (11,12) used the racemic mixture of 4,5-diaminovaleric acid, obtained by organic synthesis via the ammonolysis of 4,5-dibromovaleric acid (11,13). Consequently the question of the chirality of the participating 4,5-diaminovalerate has not thus far been addressed. This question is of considerable interest, since, remarkably, both L- and D- enantiomers of GSA are utilized by GSA aminotransferase (12). It remained to be established, therefore, whether (S)-4,5-diaminovaleric acid (formally derived from L-glutamate) or (R)-4,5-diaminovaleric acid (formally derived from D-glutamate) predominates in the catalytic process, and whether one of these diamines might possibly act as an inhibitor. Recently the utility of commercially available chiral 2-pyrrolidone-5-carboxylic acids (pyroglutamic acids) as precursors in a chirality-conserving synthesis of substrates and intermediates of the GSA aminotransferase-catalyzed reaction was recognized (14), and the preparation and NMR characterization of (S)- and (R)-4,5-diaminovaleric acids from the commercially available ethyl esters of (S)- and of (R)-2-pyrrolidone-5-carboxylic acids were reported (15). It has therefore become possi-

ble to address the question of the enantioselectivity of the *Synechococcus* GSA aminotransferase towards 4,5-diaminovalerate.

MATERIALS AND METHODS

Materials

δ-Aminolevulinic acid hydrochloride, Bis-Tris, tricine, p-dimethylaminobenzaldehyde, acetylacetone, pyridoxal phosphate, pyridoxamine phosphate, pyruvate, oxaloacetate, and α-ketoglutarate were obtained from Sigma. Other chemicals were reagent grade. (S)-4,5-diaminovaleric acid and (R)-4,5-diaminovaleric acid were synthesized from the methyl esters of (S)- and (R)-2-pyrrolidone-5-carboxylic acid (Aldrich) (15). Glutamate 1-semialdehyde, prepared by ozonolysis of 4-vinyl-4-aminobutyric acid (7), a gift from Merrell Dow Pharmaceuticals, and *Synechococcus* GSA aminotransferase, expressed in large amounts in transformed *Escherichia coli* and purified from these cells to near homogeneity (16), were gifts from Dr. C. Gamini Kannangara, Copenhagen, Denmark. 4,5-Dioxovalerate was a gift from Dr. Dieter Dörnemann, Marburg, Germany (17).

Methods

All enzyme reactions were performed in 500 µl volumes at 28°C in capped 1.5-ml Eppendorf polypropylene centrifuge tubes. ALA determination by subsequent pyrrole derivatization and color development was carried out after addition of the requisite reagents to the same tubes. Because of the increased stability of GSA with decreasing pH, all enzyme incubations were carried out at pH 6.7 (10,18). Aqueous ice-cold solutions of GSA hydrochloride were prepared just before use. Stock solutions of enzyme were diluted in 0.1 M tricine, sodium hydroxide buffer, pH 7.9 (18). Generally the reactions were started by addition of enzyme to the temperature-equilibrated incubation mixtures containing 1 mM dithiothreitol; in experiments with GSA the reactions were begun with this substrate. Reactions were stopped by addition of 10 µl of 70% perchloric acid. Pyrrole derivatization was carried out by heating at 90°C for 10 min after the addition, with thorough mixing, of 100 µl of 8 M sodium acetate buffer, pH 4.6 (18) and 60 µl of acetylacetone. A rack provided with a fasten-down cover (USA/Scientific Plastics) prevented the caps of the centrifuge tubes from popping open. At pH 4.6 acetylacetone was preferred over ethyl acetoacetate as condensing agent (19,20). Pyrrole color development was initiated at room temperature after addition of an equal volume (670 µl) of modified Ehrlich-Hg reagent (21) which, unlike modified Hg-free Ehrlich reagent (i.e. containing about 2N perchloric acid (19)), shows no sulfhydryl interference with pyrrole color development (21). (The modified Ehrlich-Hg reagent can be stored at 4°C in the dark for a least a month, while Hg-free modified Ehrlich reagent deteriorates (indicated by darkening in color) much more rapidly.) A precipitate was removed by centrifugation, and 15 - 30 min after reagent addition the absorbances were read at 553 nm. Corrections were instituted for reagent and for GSA blanks. Because of a slight deviation from linearity between absorbance and pyrrole concentration, a standard curve was used to obtain the ALA concentrations present in the 500-µl original incubation volumes; the molar absorbance at 553 nm was appr. 5.3×10^4 (21). Protein was determined by binding to Coomassie Brilliant Blue G-250 (22) (Bio-Rad Protein Assay), using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Since it is the non-chiral C1 of GSA that reacts during its enzymatic conversion to 4,5-diaminovalerate, it was reasonable to expect the chirality at C2 to be re-

Table 1. Effect of (S)- and (R)-4,5-Diaminovalerate on the Conversion of GSA to ALA

Additions		ALA formed (μM)
GSA (120 μM)		7.8
GSA (120 μM)	+ (S)-Diaminovalerate (2 μM)	27.0
	+ (S)-Diaminovalerate (4 μM)	27.3
	+ (R)-Diaminovalerate (2 μM)	10.1
	+ (R)-Diaminovalerate (4 μM)	12.4

The reactions were carried out in a water bath at 28°C. The 500-μl assay mixtures in 0.1 M Bis-Tris buffer, pH 6.7, contained dithiothreitol (1 mM), GSA aminotransferase (6.9 μg, in 0.1 M tricine, sodium hydroxide buffer, pH 7.9, diluted from stock) and further additions as indicated. The incubations were started by addition of freshly dissolved GSA hydrochloride to the temperature-equilibrated mixtures, proceeded for 6 min, were stopped by the addition of 10 μl of 70% perchloric acid and analyzed for ALA as given under "Materials and Methods".

tained. It was found, indeed, that 2 μM (S)-4,5-diaminovalerate, the metabolite expected to be formed from the physiological L-GSA, stimulated the conversion of 120 μM GSA to ALA by 246%. However, (R)-4,5-diaminovalerate also reproducibly stimulated the formation of ALA from GSA, although only by 29% under the above conditions. A doubling of the diamine concentration increased stimulation by the (S)-enantiomer only insignificantly, but doubled the stimulation by the (R)-enantiomer. It follows that saturation is achieved by lower concentrations of (S)-4,5-diaminovalerate than of (R)-4,5-diaminovalerate (Table 1). The weak participation of (R)-4,5-diaminovalerate in the DL-GSA-containing enzyme system is consistent with the observation that both the L- and D-enantiomers of GSA react with GSA aminotransferase, D-GSA being less effective (12). The GSA used here, obtained by ozonolysis of 4-vinyl-4-aminobutyric acid (7), was a mixture of the two GSA stereoisomers. To interpret the greater activation by the (S)-diamine over the (R)-diamine in the present experiments with DL-GSA, one must distinguish between the formation of 4,5-diaminovalerate from GSA, and the utilization of this compound in the second step of ALA formation. If, as is likely, only (S)-4,5-diaminovalerate is made from L-GSA, and only (R)-4,5-diaminovalerate from D-GSA, it follows that both the (S)- and the (R)-diamine can participate in the recycling of pyridoxal-*P* to pyridoxamine-*P*. The present results would indicate, therefore, that although the (S)-diamine is the preferred substrate for ALA formation, GSA aminotransferase shows a lack of strict specificity both for the enantiomeric form of GSA utilized to make the diamine, and for the stereoisomeric form of the diamine utilized to convert pyridoxal-*P* to pyridoxamine-*P*. It would follow, therefore, that experiments with L-GSA will show marked stimulation by (S)-4,5-diaminovalerate, and a slight stimulation by (R)-4,5-diaminovalerate. The slight acceleration by (R)-4,5-diaminovalerate of ALA formation from DL-GSA cannot exclude the possibility that (R)-

Table 2. Effect of 4,5-dioxovalerate, pyruvate, oxaloacetate, α-ketoglutarate on ALA formation from (S)-4,5-diaminovalerate

Additions (120 μM each)			ALA formed (μM)
(S)-4,5-diaminovalerate			n.r.
(S)-4,5-diaminovalerate	+	4,5-dioxovalerate	5.7
	+	pyruvate	n.r.
	+	oxaloacetate	n.r.
	+	α-ketoglutarate	n.r.

The reactions were carried out at 28°C in 0.1 M Bis-Tris buffer, pH 6.7, containing dithiothreitol (1 mM), and GSA aminotransferase as described in Table 1, as well as pyridoxal-*P* and pyridoxamine-*P* (2 μM each), and additions as indicated. Incubations were carried out for 30 min. ALA concentrations given correspond to those formed in 30 min incubation. The abbreviation "n.r." stands for "no detectable reaction", i.e. <0.9 μM ALA formed.

4,5-diaminovalerate inhibits the formation of (S)-4,5-diaminovalerate from L-GSA, since such an inhibition would be compensated by a greater activating effect of (R)-4,5-diaminovalerate on the conversion of pyridoxal-*P* to pyridoxamine-*P*. From a practical point of view the potentiation of ALA formation by (S)-4,5-diaminovalerate may in the future make it advisable routinely to add this compound to enzyme extracts examined for the presence of the C_5 pathway of ALA formation, particularly since the rate-limiting step from glutamate to ALA may not be the same in all systems.

The availability of (S)-4,5-diaminovalerate made it of interest to investigate the basis of the inhibition by 4,5-dioxovalerate of the transformation of GSA to ALA (11,12). Since this transformation occurs in two steps, the dioxo compound could inhibit either the conversion of GSA to 4,5-diaminovalerate, or the conversion of 4,5-diaminovalerate to ALA. To distinguish between these alternatives the possible reaction between (S)-4,5-diaminovalerate and 4,5-dioxovalerate was investigated. It was found that, while enzyme incubation of the (S)-diamine alone led to no measurable ALA, enzyme incubation with an (S)-4,5-diaminovalerate, 4,5-dioxovalerate mixture did produce ALA (Table 2), although at a substantially lower rate than from GSA (Table 1). It is hence likely that it is the formation of the diamine from GSA, the first step in the transformation of GSA to ALA, that is inhibited by the dioxo compound. The nature of this inhibition has not been determined.

The reaction between (S)-4,5-diaminovalerate and and 4,5-dioxovalerate resembles a conventional transamination: the (S)-diamine acts as amino donor and the dioxo compound as amino acceptor. In order to investigate the specificity of the participation of 4,5-dioxovalerate as amino acceptor from (S)-4,5-diaminovalerate,

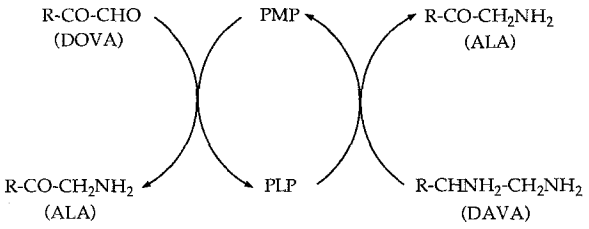

Fig. 2. 4,5-Dioxovalerate (DOVA) can take the place of glutamate 1-semialdehyde (GSA) in Fig. 1A, leading to the formation of 2 molecules of δ-aminolevulinate (ALA) per cycling between enzyme-bound pyridoxamine-*P* (PMP) and enzyme-bound pyridoxal-*P* (PLP).

the possible activity of the substances that normally function as amino acceptors in aminotransferase reactions was tested. Neither pyruvate, oxaloacetate, nor α-ketoglutarate participated in the formation of ALA from (S)-4,5-diaminovalerate (Table 2). GSA aminotransferase would appear, therefore, to belong to the relatively small group of aminotransferases that utilize only one or a very limited number of amino group acceptors (23-32); this enzyme appears, moreover, to be a very rare example of an aminotransferase that does not react with any of the above three α-keto acids. Only one other case of this kind appears to have been reported before (26). (It turns out that among the various acceptor-specific aminotransferases only one catalyzes a transamination with the α-amino group of an α-amino acid (26).) The ability, though limited, of 4,5-dioxovalerate to take the place of GSA as an amino acceptor from pyridoxamine-*P* – presumably, as for GSA, at the terminal carbon – almost certainly does not indicate a physiological role for the dioxo compound; it is more probable that the ability of the dioxo compound to function as a poor amino acceptor from enzyme-bound pyridoxamine-*P* (Fig. 2) is a consequence of the structural similarity of 4,5-dioxovalerate to GSA. It may be noted that the stoichiometry of ALA formation attending the reaction between 4,5-dioxovalerate and (S)-4,5-diaminovalerate differs from that which attends the transformation of GSA to ALA. In the conversion of GSA to ALA via the diamine only *one* ALA is formed per pyridoxal-*P* ⟶ pyridoxamine-*P* ⟶ pyridoxal-*P* cycle. In the case of the reaction between the dioxo compound and the diamine, however, an ALA molecule is generated both in the transfer of an amino group from pyridoxamine-*P* to the dioxo compound and in the transfer of an amino group from the (S)-diamine to the resulting pyridoxal-*P*, so that *two* ALAs are formed per cycle (Fig. 2). The slow rate of ALA formation when the dioxo compound and the (S)-diamine react, due to the slow reaction between 4,5-dioxovalerate and pyridoxamine-*P* as against the faster reaction between GSA and pyridoxamine-*P*, hence should be divided by 2 for a more appropriate comparison with the rate of ALA formation from GSA and (S)-4,5-diaminovalerate.

In conventional aminotransferase systems two different α-amino acids participate, one α-amino acid resulting from the transfer of an amino group from pyridoxamine-*P* to a given α-keto acid, the other α-amino acid functioning as amino donor to the ensuing pyridoxal-*P*. In such a system the two half-reactions treat the substrates identically: one is confined to the gain or loss of amino or oxo groups at α-carbons. In the two-step GSA to ALA system, however, 4,5-diaminovalerate manifests two different modes of participation, since two different amino groups are involved: a primary amino group is gained in the first step (at C5), and a secondary amino group is lost in the second step (at C4). It is the participation of these two distinct amino groups of 4,5-diaminovalerate that enables the net reversal of amino and oxo substituents, necessary for the transformation of GSA to ALA, to occur. In the two roles of 4,5-diaminovalerate in the present system, as product and as substrate, different parts of the diamine are recognized by the enzyme. In a strictly formal sense, therefore, the present aminotransferase treats 4,5-diaminovalerate in the two instances of its participation as though it were two distinct molecules, so that the general prerequisite for aminotransferases, the participation of two distinct amino-carrying participants, is maintained.

ACKNOWLEDGMENTS

This work was supported by the Louis Block Fund of the University of Chicago (to H.C.F.) and by Grant GM-11973 from the National Institutes of Health (to B.F.). We thank Dr. C. Gamini Kannangara for making articles available before publication, and Dr. John Westley for a critical reading of the manuscript.

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