Active Site-Directed Inactivation of Cystathionine γ-Synthetase and Glutamic Pyruvic Transaminase by Propargylglycine

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Summary: The acetylenic amino acid propargylglycine (2-amino-4-pentynoate) irreversibly inactivates two pyridoxal-P dependent enzymes which generate substrate-derived beta carbanions during catalysis.

Introduction: We have recently argued that acetylenic substrate analogues should be specific, irreversible inactivators of enzymes which, during catalysis on such analogues, can abstract a proton and generate a carbanion adjacent to the acetylenic linkage (1). Propargylic rearrangement of the acetylenic anion could yield an allenic anion as nucleophile or, on protonation, an electrophilic allene. Either species could covalently modify active site amino acids (2-4) or tightly bound coenzymes (5-7).

The acetylenic amino acid propargylglycine (2-amino-4-pentynoate) is a potent inactivator of the pyridoxal-P dependent γ-cystathionase from mammalian liver (1). While this enzyme must, at an early step, generate a substrate α-carbanion in common with almost all pyridoxal enzymes, the electronic requirements for the unusual γ-elimination require obligate β-carbanion formation as well. With propargylglycine, enzymatic formation of an α-carbanion leaves the acetylenic functionality still insulated and unreactive with the great majority of Vitamin B_6-dependent enzymes; but subsequent β-carbanion formation by γ-cystathionase activates this acetylenic amino acid for propargylic rearrangement to a conjugated allene, now susceptible to nucleophilic attack, possibly by the ubiquitous active site lysine ε-amino group.

In this communication we report the inactivating effects of propargylglycine on two other pyridoxal-P dependent enzymes, cystathionine γ-synthetase from Salmonella typhimurium and, less expectedly, glutamic pyruvic transaminase from pig heart.

Materials and Methods: D,L-homoserine and allylglycine were purchased from Sigma. O-succinyl-D,L-homoserine was prepared by a literature

procedure (8). D,L-propargylglycine synthesis has been described (1). L-2-amino-4-methoxy-trans-3-butenoate was a gift from H.R. Kaback, The Roche Institute of Molecular Biology. Cystathionine γ-synthetase from Salmonella typhimurium me-A-15 was purified through step 5 of the procedure of Kaplan and Guggenheim (8) which in our hands yielded enzyme with a specific activity of 0.2U/mg, rather than the 4.5U/mg reported (8), representing an 8-fold purification, using the γ-elimination assay coupled to lactic dehydrogenase. Glutamic pyruvic transaminase (GPT) at 85 U/mg and glutamic oxalacetic transaminase (GOT) at 280 U/mg were from Sigma.

Inactivation experiments with cystathionine γ-synthetase of the type described in figure 1 involved the following protocol. At time zero, 25 µl of an appropriate propargylglycine solution was added to 225 µl of solution containing 50 mM KPPi, pH 8.2, and 0.5 mg of the partially purified Salmonella enzyme at 37°. At intervals, 50 µl aliquots were removed and assayed by dilution into cuvettes containing 1 ml of 5 mM O-succinyl homoserine, 50 mM KPPi, pH 8.2, 0.3 mM NADH, and 0.2 mg muscle lactate dehydrogenase, permitting continuous assay of 2-ketobutyrate formation. In similar experiments with GPT, 10 µg GPT was added to 200 µl of 80 mM Tric-HCl, pH 8, 37°, containing varying amounts of propargylglycine. At intervals, 25 µl aliquots were removed, then added into a cuvette at 25° containing 200 mM L-alanine, 20 mM 2-ketoglutarate, 80 mM Tris-HCl, pH 8, 0.3 mM NADH, and 10 µg lactate dehydrogenase to assay pyruvate production. A similar protocol was used for experiments with GOT.

Results:

Cystathionine γ-Synthetase: Cystathionine γ-synthetase, involved in microbial and plant biosynthesis of methionine, catalyzes a γ-replacement on an O-acyl-homoserine with cysteine (or other thiols) to form cystathionine and a liberated acyl moiety. In the absence of such thiol acceptors, the enzyme will also perform a γ-elimination to give 2-ketobutyrate and the free acyl anion. A β-carbanion intermediate is invoked in both these catalytic aspects of the enzyme. Thus, we were tempted to test propargylglycine as a suicide substrate for the synthetase.

The data of figure 1 show the rates of irreversible inactivation of the partially purified Salmonella enzyme with different concentrations of acetylenic amino acid. The half time for inactivation at 1 mM propargylglycine is 4 minutes. Also indicated is the marked protection afforded by 10 mM cystathionine, consistent with the idea that the inactivation occurs specifically at the active site. The irreversible nature of the inactivation was confirmed by assay after exhaustive dialysis

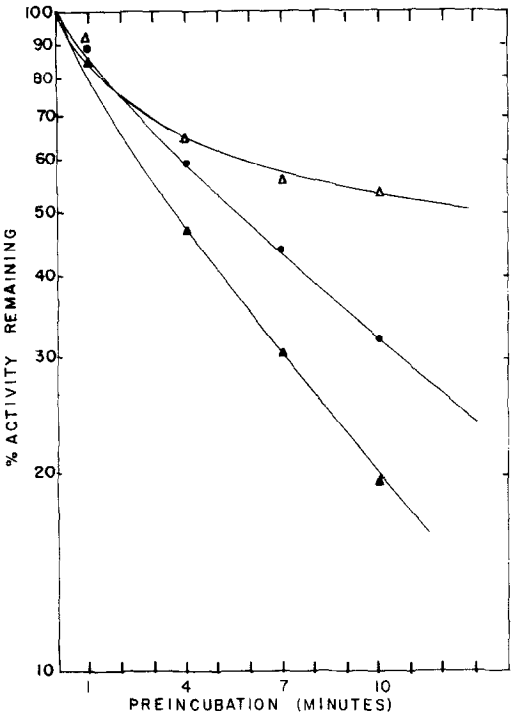

Figure 1. Time Course of Inactivation of Cystathionine γ-Synthetase with Propargylglycine. Incubations were performed as described in the text. Propargylglycine concentrations were: 0.4 mM ●-● ; 1.0 mM ▲-▲ ; 1.0 mM plus 10 mM allocystathionine Δ-Δ . D,L-allylglycine (5 mM) produced no inactivation in 10 minutes.

against buffer containing pyridoxal-P; 80% of the activity was permanently lost while control enzyme samples were completely active. The recovery of 20% of the activity may be related to the lability of the covalent modification. This will be checked with radioactive propargylglycine. Further purification of the enzyme to homogeneity (8) should allow determination of stoichiometry of inactivator attachment, spectroscopic evaluation of pyridoxal coenzyme involvement, and identification of any modified active site amino acids.

Transaminases: We have also examined the inactivating propensity of propargylglycine towards two mammalian transaminases, pig heart glutamic pyruvic transaminase (GPT) and pig heart glutamic oxalacetic transaminase (GOT). It has generally been supposed that amino acid α-carbanions but not β-carbanions are intermediates in such transaminations. However, Babu and Johnson (9) have recently reported, from NMR experiments in D_2O,

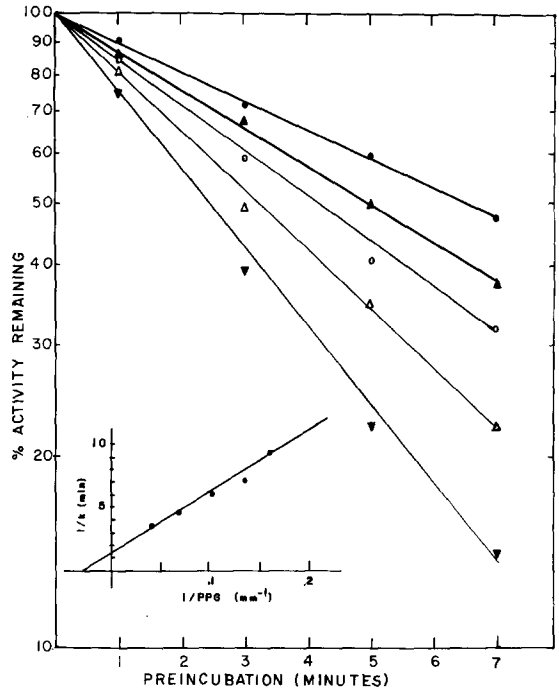

Figure 2. Time Course of Inactivation of Glutamic Pyruvic Transaminase with Propargylglycine. Propargylglycine concentrations were: 6.25 mM ●-● ; 7.5 mM ▲-▲ ; 10 mM o-o ; 15 mM Δ-Δ ; 25 mM ▼-▼ . As noted in figure 1 legend, allylglycine (50 mM) caused no loss of activity here in 7 minutes. The inset is a double reciprocal plot of D,L-propargylglycine concentration vs. pseudo first order rate of inactivation.

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that GPT will exchange the beta hydrogens of L-alanine, 9 Units of enzyme causing complete loss of the methyl signal of 120 umoles of substrate in 15 minutes. Purportedly, GOT carries out the same exchange but, "at a much slower rate that GPT (9)". These data indicate that GPT, and perhaps GOT, can form β-carbanion equivalents with appropriate substrates even though no mechanistic role in transamination can be assigned.

The data of figure 2 show the pseudo-first order rates of inactivation of GPT with differing amounts of propargylglycine; for instance, 5 mM causes 50% inactivation in 7 minutes at 37°. The inset shows a reciprocal plot from which a K_m of 38 mM can be extracted as well as a rate constant for inactivation of 0.8 min^{-1}. This is consistent with initial formation of a Michaelis complex followed by a second, catalytic step leading to inactive enzyme. Again, substrate affords protection over the 10 minute inactivation period.

The inactivation process remains fully irreversible after gel fil-

tration to remove excess substrate. Repetition of the inactivation with 1000 Units of enzyme to 98% inactivation, followed by gel filtration produced an inactive enzyme in which the absorption spectrum of the bound coenzyme had been altered: the 412 nm peak due to pyridoxal-P had disappeared and been replaced by an absorbance maximum at 325 nm, suggesting the pyridoxamine form of the coenzyme. This would be expected if an active site residue attacked a 2-imino-3,4-pentadienyl intermediate followed by Schiff base hydrolysis to the amino form of the coenzyme and an alkylated active site residue. Purification of the commercial enzyme to homogeneity will permit determination of inactivation stoichiometry and whether coenzyme modification has in fact occurred.

When the other transaminase, GOT, was examined, 100 mM propargylglycine had no inactivating effect during a 10 minute treatment; although we have yet to determine a K_i for propargylglycine with GOT, this insensitivity may relate to the different ratios at which GPT and GOT generate substrate carbanions. Rando (10) has recently reported that GOT is irreversibly blocked by the bacterial metabolite L-2-amino-4-methoxy-3-butenoate, presumably by Michael addition of an active site nucleophile to the conjugated, transaminated product. We likewise find GOT to be susceptible; 10 mM alkenoic amino acid produces 50% inactivation in one minute at 37°. Contrarily, GPT is inert to this reagent, with no detectable loss of activity in a 30 minute incubation under the same conditions.

Discussion: The irreversible loss of cystathionine γ-synthetase activity caused by propargylglycine suggests this enzyme probably abstracts the α and β hydrogens of the acetylenic amino acid prior to formation of the inactivating species. That this bacterial enzyme and the mammalian γ-cystathionase are targets of this compound might be expected from their mechanistic similarities (11). In 1949 Gershon et al. (12) noted growth inhibition of yeast and *E. coli* by propargylglycine. We have now confirmed these observations with *E. coli*, *S. typhimurium*, and *B. subtilis*, all known to contain cystathionine γ-synthetase which is probably the major if not sole target in these bacteria.

The ability of propargylglycine to kill GPT under conditions where GOT is unaffected is consistent with the relative capacity of the transaminases to labilize substrate β hydrogens. Coupled with exactly the reverse susceptibility to aminomethoxybutenoate as an inactivator, one now has available at least *in vitro* two suicide substrates with mutually exclusive action on these two metabolically central transaminases. We

are investigating potential in vivo selectivity and the molecular bases for such differential specificities. It will be of interest to examine for loss of catalytic activity other enzymes which can recognize propargylglycine; loss of activity should be diagnostic of the catalytic capacity of that enzyme to form β-carbanionic species.

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References

1. R. Abeles and C. Walsh (1973) J. Amer. Chem. Soc., 95, 6124.
2. K. Bloch (1971) in "The Enzymes". P.D. Boyer (Ed.), 3rd Edition, Volume V, pp 441ff, Academic Press, N.Y.N.Y.
3. R.C. Hevey, J. Babson, A.L. Maycock, and R. Abeles (1973), J. Amer. Chem. Soc., 95, 6125.
4. P.C. Holland, M.G. Clark, and D,P. Boxham (1973) Biochemistry, 12, 3309.
5. C. Walsh, A. Schonbrunn, O. Lockrdige, V. Massey, and R. Abeles (1972), J. Biol. Chem., 247, 6004.
6. R. Rando (1973) J. Amer. Chem. Soc., 95, 4438.
7. H.Y.K. Chuang, D.R. Patek, and L. Hellerman (1974) J. Biol. Chem., 249, 2381.
8. M. Kaplan and S. Guggenheim in "Methods in Enzymology", H. Tabor and C.W. Tabor (Eds.), Volume XVII, Part B, p. 425, 1971, Academic Press, N.Y.N.Y.
9. U.M. Babu and R.B. Johnston (1974) Biochem. Biophys. Res. Commun., 58, 460.
10. R. Rando (1974) Nature, 250, 586.
11. L. Davis and D.E. Metzler in "The Enzymes", P.D. Boyer (Ed.) 3rd Edition, Volume VII, p. 33, 1972. Academic Press, N.Y.N.Y.
12. H. Gershon, J. Meek, K. Dittmer (1949) J. Amer. Chem. Soc., 71, 3573.