INACTIVATION OF PYRIDOXAL 5′-PHOSPHATE-DEPENDENT ENZYMES BY 5-NITRO-L-NORVALINE, AN ANALOG OF L-GLUTAMATE

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Received 26 February 1981

1. Introduction

Substrate analogs bearing ionizable nitro groups in place of carboxylate groups have proven to be interesting inhibitors of enzymes [1–5]. For instance, 2-nitropropane (which is isoelectronic with the alanine zwitterion) and the toxic antibiotic 3-nitropropionate (which is isoelectronic with succinate) irreversibly inactivate the flavin-dependent enzymes D-amino acid oxidase and succinate dehydrogenase, respectively, in mechanistically distinct 'suicide' reactions [3]. The nitronates obtained upon deprotonation of aliphatic nitro compounds generally bind well to enzymes which act on corresponding carboxylic compounds. In fact, in special cases in which the nitronates function as 'transition-state analogs', they bind by orders of magnitude more tightly than the carboxylic substrates [4,5].

Here, we report that several pyridoxal 5′-phosphate-dependent enzymes are subject to inactivation by a nitro analog of their substrate glutamate. Although 5-nitro-L-norvaline (fig.2) sterically resembles L-2-aminoadipic acid somewhat more closely than it resembles L-glutamic acid, the lower homolog of 5-nitro-L-norvaline is an unknown and probably unstable [6] compound. However, the nitronate form of 5-nitro-L-norvaline exhibits nearly the same intramolecular charge separation as occurs in the L-glutamate anionic species; and 5-nitro-L-norvaline is accepted into the active sites of alanine aminotransferase (EC 2.6.1.2), aspartate aminotransferase (EC 2.6.1.1), and 4-aminobutyrate aminotransferase (EC 2.6.1.19), all of which act on L-glutamate.

2. Materials and methods

5-Nitro-L-norvaline (a gift from Dr W. Keller-Schierlein) [7] and 3-nitro-D,L-alanine (a gift from Dr D. J. T. Porter) [5] were synthesized as described. 4-Aminobutyrate aminotransferase and succinate semialdehyde dehydrogenase were from *Pseudomonas fluorescens*. The other aminotransferases as well as lactate and malate dehydrogenases were the cytosolic enzymes from porcine heart. The enzymes and cofactors were products of the Sigma (St Louis MO). After dialysis against phosphate buffer, the aminotransferases were not stimulated by additional pyridoxal 5′-phosphate.

The aminotransferase activities were assayed by the dehydrogenase-coupled method [8]. One unit of aminotransferase was taken to generate 1.0 μmol product/min from 50 mM substrate (L-alanine, L-aspartate, or 4-aminobutyrate) in the presence of 10 mM 2-oxoglutarate, 1.0 mM EDTA, and 100 mM Tris–HCl at pH 8.0 and 25°C.

3. Results

Representative time courses for the inactivation of aminotransferases by 5-nitro-L-norvaline are shown in fig.1. The inactivation reproducibly does not proceed in a first-order exponential manner. The inactivation does not proceed to completion at low 5-nitro-L-norvaline concentrations, and the incompleteness of the inactivation is not mitigated by the presence of 1.0 mM 2-oxoglutarate. The incompleteness is thus not secondary to the conversion of the enzymes to their pyridoxamino states. One reason [8] that the loss of activity is not a simple first-order function of time is that the inactivation is, in part, slowly reversible. After nearly complete inactivation of aspartate aminotransferase by 5-nitro-L-norvaline, 96% of the activity is recovered within 8.0 h of dialysis against

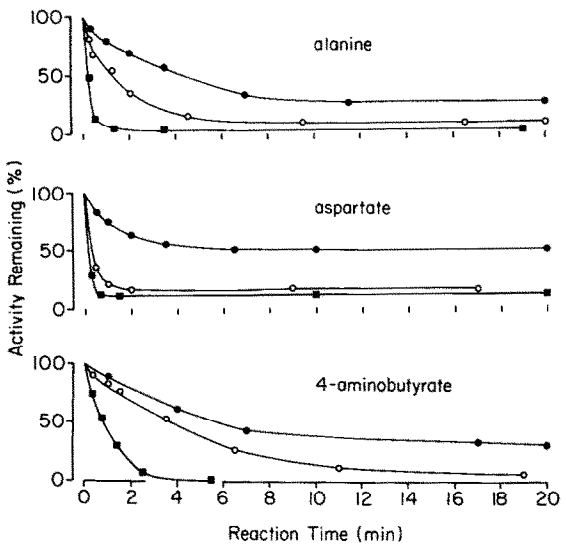

Fig.1. Inactivation of aminotransferase by 5-nitro-L-norvaline. The indicated enzymes (~1 unit/ml) were incubated with 5-nitro-L-norvaline (5.0, 20 and 100 μM) in 100 mM potassium phosphate buffer at pH 7.4 and 25°C, and small aliquots of the reaction mixture were periodically diluted 100-fold and tested for transaminase activity. Inactivation does not require the presence of an oxoacid substrate and can be completely prevented by the presence of 50 mM L-glutamate.

10 mM potassium phosphate buffer at pH 7.4 and 4°C. However, only 36% and 8%, respectively, of the alanine and 4-aminobutyrate aminotransferase activities are recovered, and these yields are not increased by treatment with 10 mM D,L-dithiothreitol and 0.1 mM pyridoxal 5′-phosphate during more-prolonged dialysis. Samples of the enzymes not treated with 5-nitro-L-norvaline retain over 90% of their activity when subjected to dialysis for 24 h. The kinetically complex inactivation thus has both slowly reversible and irreversible features, and kinetic analysis is further complicated by the observation that some totally irreversible active-site directed inactivators of these enzymes are known to react in a markedly biphasic manner [9].

The inactivation is not a non-specific effect of the nitro group. Thus, neither 10 mM nitroethane nor its potassium salt inactivate the enzymes under the conditions in fig.1. The enzymes are very slowly and irreversibly inactivated by 10 mM 3-nitro-D,L-alanine, but that reaction probably proceeds by a different mechanism analogous to that of the suicide substrate 3-fluoroalanine [1].

4. Discussion

The anticipated reaction mechanism which prompted this investigation is outlined in fig.2. The ketimine adduct of 5-nitro-L-norvaline with pyridoxal 5′-phosphate is expected to cyclize in a reaction similar to the aldol condensation. Model reactions for the proposed cyclization include the slow reactions of pyridoxal 5′-phosphate with dopa or with histidine to afford 6-membered cyclic adducts [10,11]. The cyclization of 5-nitro-L-norvaline is probably slowly reversible, but the initially-generated 6-membered structure shown in fig.2 may irreversibly eliminate inorganic nitrite because of the carbanion-stabilizing pyridinium substituent. Other derivatives of 1-amino-4-nitrobutane in addition to 5-nitro-L-norvaline ought to similarly inactivate those pyridoxal 5′-phosphate-dependent enzymes for which the substrates are resembled. Similarly, derivatives of 1-amino-3-nitropropane ought to inactivate such enzymes by forming 5-membered cyclic adducts with the coenzyme. Thus, advances in the organic synthesis of nitroalkylamines may permit their use as selective inactivators of pharmacologically interesting targets [1] including 4-ami-

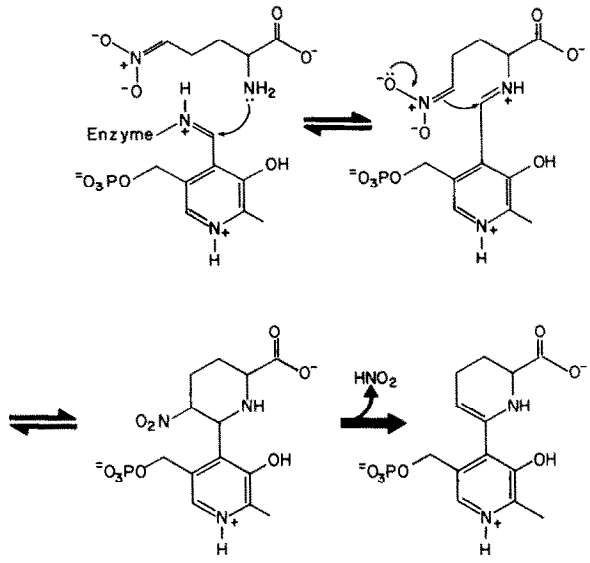

Fig.2. Probable mechanism by which pyridoxal 5′-phosphate-dependent enzymes are inactivated by 5-nitro-L-norvaline. A metastable 6-membered cyclic adduct is generated in a slowly reversible reaction, but the adduct may irreversibly eliminate inorganic nitrite [2] because of the influence of the pyridinium ring.

nobutyrate aminotransferase, L-dopa decarboxylase, L-histidine decarboxylase, L-serine transhydroxymethylase, the microbial enzymes acting on D-amino acids, and the amine oxidases possessing pyridoxal-like prosthetic groups.

Acknowledgements

This study was supported by research grant GM 11040 from the National Institutes of Health. T. A. A. is supported by the Medical Scientist Training Program, GM 07170, National Institutes of Health. The 5-nitro-L-norvaline used in this study was a generous gift from Dr W. Keller-Schierlein, Eidgenössische Technische Hochschule, Zürich.

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