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CHEMICAL MODIFICATION OF BACTERIAL 4-AMINOBUTYRATE AMINOTRANSFERASE BY PHENYLGLYOXAL

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4-Aminobutyrate aminotransferase (EC 2.6.1.19), obtained from *Pseudomonas fluorescens*, was irreversibly inhibited by phenylglyoxal, a reagent that specifically modifies arginyl residues. The inactivation appeared to be the result of a simple, bimolecular reaction since no evidence of a reversible complex between inhibitor and enzyme emerged. The second-order rate constant was 0.221 ± 0.077 M^{-1} sec^{-1}. The concentration of either substrate had no effect on the inhibition, but an increase in the concentration of pyridoxal 5′-phosphate reduced the rate of inactivation by phenylglyoxal. The data are consistent with the modification of amino acid residues at the cofactor binding site on the enzyme.

KEY WORDS: GABA aminotransferase, inactivation, phenylglyoxal, arginyl residues, pyridoxal 5′-phosphate, *Pseudomonas fluorescens*

INTRODUCTION

With the assistance of pyridoxal 5′-phosphate as cofactor, 4-aminobutyrate (GABA) aminotransferase (EC 2.6.1.19) is responsible for the catalytic conversion of GABA and α-ketoglutarate to succinic semialdehyde and glutamate, respectively.[1] In the mammalian nervous system this pathway represents the metabolic degradation of GABA which functions as an inhibitory neurotransmitter.[2] In plants and microorganisms, however, the significance of this metabolic route is poorly understood.

Mammalian GABA aminotransferase has been treated with several residue-specific reagents which produced an inactivation of the enzyme. Inhibition by such compounds as 5,5-dithiobis-(2-dinitrobenzoate) or *p*-chloromercuribenzoate[3] suggests the presence of essential cysteinyl residues. Further, inactivation by ortho-phthalaldehyde or a pyridoxal 5′-phosphate analogue has revealed the involvement of lysyl residues in catalytic activity at the α-ketoglutarate binding site.[4,5] Finally, phenylglyoxal inhibits the enzyme from mouse brain, which inhibition can be reduced in the presence of pyridoxal 5′-phosphate. Since phenylglyoxal reacts with arginyl residues,[6] this observation indicates that arginine at the cofactor binding site appears to have a

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functional role in enzyme activity.[7] Recently the enzyme from pig liver and rat brain has been cloned and sequenced.[8–10] The sequences for the enzyme from several microorganisms have also been published.[11–13] From such studies it has emerged that the mammalian and the bacterial enzymes differ in certain ways. For example, the sequence identity of liver GABA aminotransferase is only 22% with that from *E. coli*,[9] and the molecular mass of recombinant pig brain enzyme is 28% larger than the bacterial enzyme.[14]

The enzyme from *Pseudomonas fluorescens* has not yet been studied in detail; however, recent work from this laboratory has shown that GABA aminotransferase isolated from this microorganism is susceptible to inactivation by 3-bromopyruvate acting as an affinity label.[15] Since 3-bromopyruvate is known to be capable of forming covalent bonds with sulfhydryl groups,[16–19] GABA aminotransferase most likely contains essential cysteinyl residues. Indeed, GABA aminotransferase from *Streptomyces griseus* is inhibited by several sulfhydryl-group reagent.[20]

The present experiments were carried out to further study the effects of modifying essential amino acid residues of GABA aminotransferase from *Pseudomonas fluorescens*. The activity of the enzyme was followed in the presence of different concentrations of phenylglyoxal, the arginine-specific reagent. This type of methodology, which employs an irreversible inhibitor during the enzyme catalytic process, has been studied by Tsou.[21]

MATERIALS AND METHODS

Source of Enzyme and Chemicals

Partially purified GABA aminotransferase, also containing succinic semialdehyde dehydrogenase, was purchased from Sigma Chemical Company, St. Louis. Specific activity was 1.5 units per mg. protein.

Phenylglyoxal, GABA, α-ketoglutarate, succinic semialdehyde, pyridoxal 5′-phosphate and NAD were also obtained from Sigma Chemical Company.

Assay of Enzyme Activity

The measurement of the activity of GABA aminotransferase took advantage of a coupled assay. The product of the first reaction, succinic semialdehyde, is oxidized to succinate in the second reaction with the concommitant production of NADH. The presence of the latter was detected in a Gilford model 2400S spectrophotometer at a wavelength of 340 nm.

For a typical assay the concentration of both GABA and α-ketoglutarate was 2 mM. NAD^+ and pyridoxal 5′-phosphate were present at 5 mM and 0.05 mM, respectively. The reaction was monitored at 30°C in 50 mM Tris-HCl buffer, pH 8.6. Incubations were started by the addition of a small volume of a pre-warmed solution of buffer and enzyme.

In another series of experiments the activity of succinic semialdehyde dehydrogenase was measured. For this, succinic semialdehyde (10 μM) was substituted for

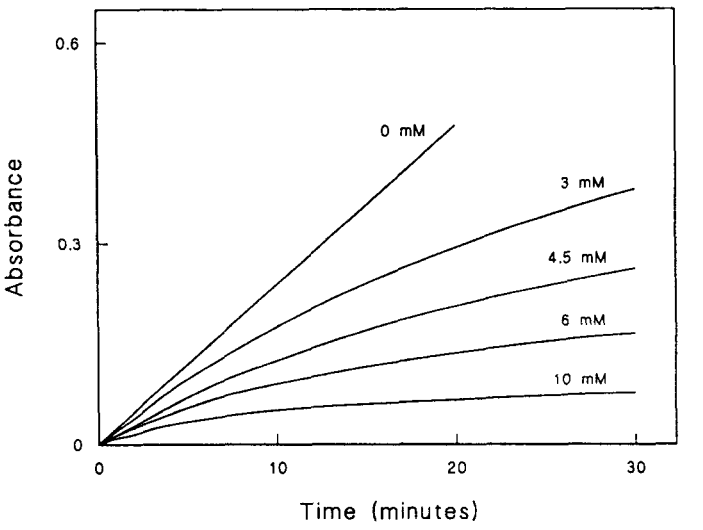

FIGURE 1 Time course of NADH production under conditions described in Materials and Methods section. (A) Enzyme without inhibitor; (B,C,D, and E) in the presence of 3, 4.5, 6, and 10 mM phenylglyoxal, respectively.

GABA and α-ketoglutarate in the above assay system, and the formation of NADH was monitored in the usual manner. Previous experiments in this laboratory have shown that succinic semialdehyde has a K_m of 4.5 μM for its enzyme.

RESULTS

Influence of Phenylglyoxal on GABA Aminotransferase Activity

Enzyme activity was followed as a function of time in the absence and presence of several different phenylglyoxal concentrations. Figure 1 demonstrates that activity was linear with no inhibitor present. The presence of phenylglyoxal inhibited enzyme activity in a concentration-dependent manner, i.e. the maximum absorbance reached was related to the amount of phenylglyoxal present during the transamination reaction. The shape of the progress curves suggests that the inhibition is irreversible. It has been demonstrated[22] that in the presence of both substrate and inhibitor the appearance of product with time can be described by the expression:

$$[P] = [P]_\infty (1 - e^{-A[I]t}) \quad (1)$$

where $[P]_t$ and $[P]_\infty$ represent the product concentration at time t and at t infinity. The apparent second-order rate constant, A, is for the reduction in enzyme activity and [I] is the concentration of inactivator. For this expression to be valid, substrate

concentrations and [I] must hardly decrease during the reaction. Equation 1 predicts that [P] approaches a constant value when t is at infinity, and the data in Figure 1 are consistent with this equation.

Tian and Tsou[22] have shown that:

$$\ln([P]_\infty, -[P]_t) = \ln[P]_\infty - A[I]t \quad (2)$$

A plot of $\ln([P]_\infty - [P]_t)$ against t should therefore give a straight line whose slope is $-A[I]$. This would be the observed rate of inactivation (k_{obs}). Figure 2 shows the results of such plots from the data in Figure 1. Straight lines resulted for each inhibitor concentration and k_{obs} increased with increasing amounts of phenylglyoxal.

When k_{obs} was plotted against [I], a linear relationship between the two parameters was obvious (Figure 3), indicating the absence of saturation kinetics during the inactivation process. A plot of the data expressed as reciprocal values (Figure 3, inset) further illustrates the linear relationship. Some of the values making up the y axis of Figure 3 were obtained from slopes not shown in the previous figure in order to simplify that figure. The mean value for the second-order rate constant from three experiments was calculated as $0.221 \pm 0.077\ M^{-1}\ sec^{-1}$, and represents the slope of the line.

Influence of Substrate and Cofactor Concentrations on Inactivation

In the presence of inhibitor, enzyme activity was monitored at a constant level of α-ketoglutarate and cofactor but at increasing concentrations of GABA. Progress curves, similar to those in Figure 1, were obtained. As described earlier, k_{obs} values were calculated from a graph of $\ln([P]_\infty - [P_t])$ against time. Substrate concentration was then plotted against 1/A (Figure 4). A straight line parallel to the y axis resulted, demonstrating that GABA was unable to affect the rate of inactivation brought about by 5 mM phenylglyoxal. Similarly, GABA and pyridoxal 5′-phosphate concentrations were held at 2 mM and 0.5 mM, respectively but α-ketoglutarate levels were systematically increased. Again, the rate of inactivation was unaltered. However, the rate of inactivation was reduced if pyridoxal 5′-phosphate concentrations were increased but the concentration of both substrates was held constant (Figure 5).

Influence of Phenylglyoxal on Succinic Semialdehyde Dehydrogenase Activity

When the activity of succinic semialdehyde dehydrogenase was monitored over a 15-minute period in the absence or presence of 10 mM phenylglyoxal, no sign of an effect on the rate of NADH production was observed (data not shown).

DISCUSSION

These experiments were carried out with phenylglyoxal present during the assay of the enzyme. This procedure, which has been extensively investigated by Tsou,[21] offers

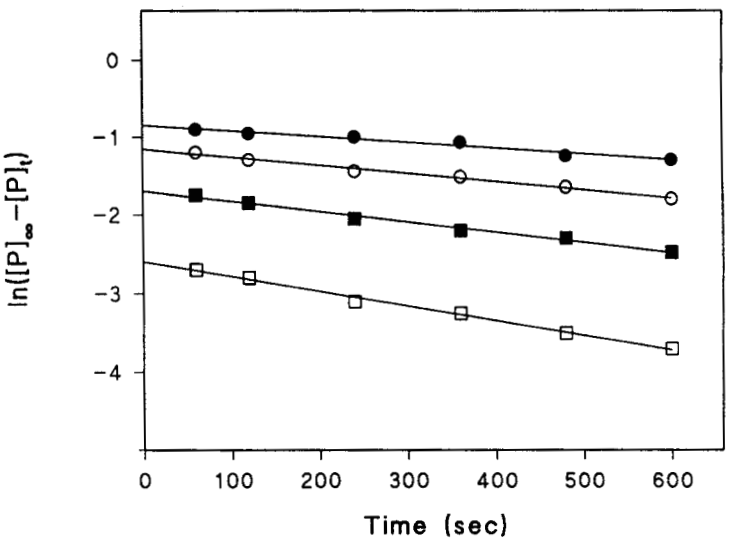

FIGURE 2 Enzyme inactivation rates using data from Figure 1. The slope of the lines represent k_{obs} and equals −A[I]. The concentrations of phenylglyoxal (3 mM, 4.5 mM, 6 mM and 10 mM) are represented by closed circles, open circles, closed squares, and open squares, respectively.

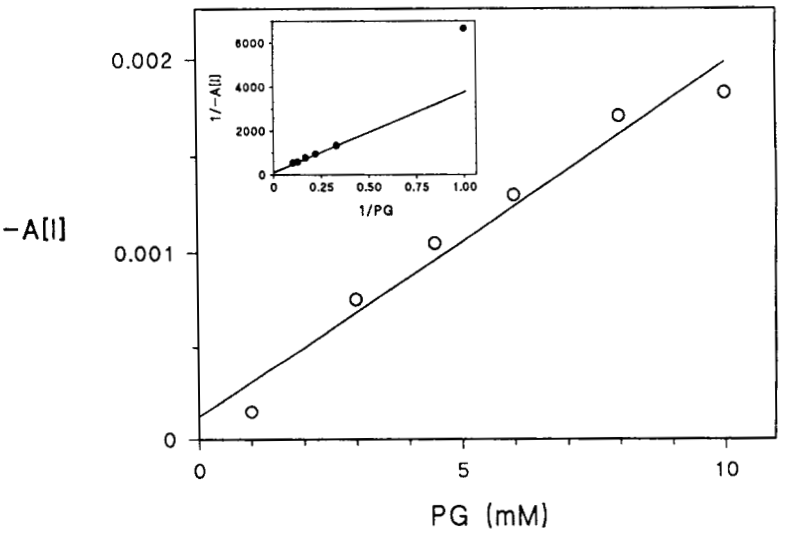

FIGURE 3 Relationship between rate of inactivation (k_{obs}) and phenylglyoxal concentration. Units of the ordinate (k_{obs}) = sec^{-1}. Inset shows the data plotted with reciprocal values. Standard errors for each data point were smaller than the symbols.

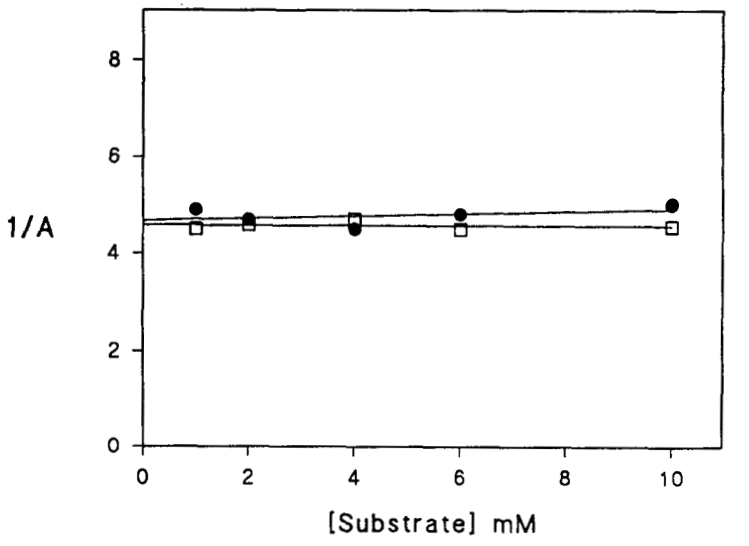

FIGURE 4 Effects of substrate concentration on apparent second-order rate constants (units = M^{-1} sec^{-1}). Standard errors for each data point were smaller than the symbols. ● = GABA; □ = α-ketoglutarate.

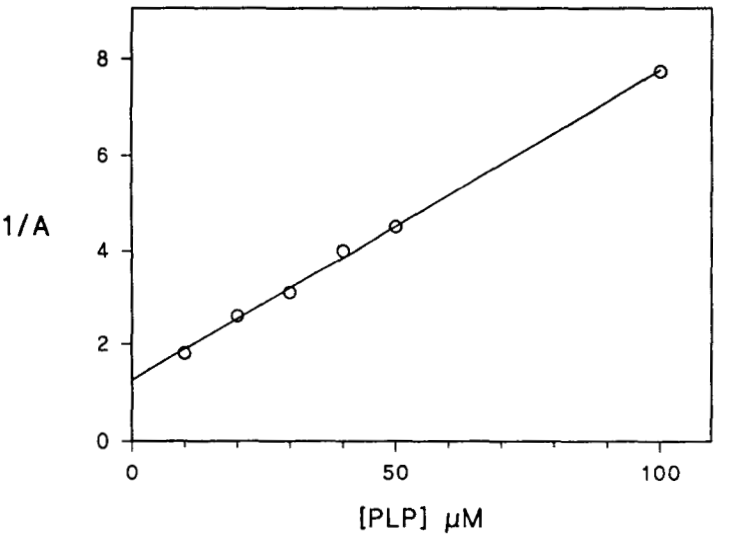

FIGURE 5 Effects of cofactor concentration on apparent second-order rate constants (units = M^{-1} sec^{-1}). Standard errors for each data point were smaller than the symbols.

advantages over methods in which enzyme is first exposed to inhibitor and then, after removal of the inhibitor, the residual enzyme activity is assayed. Far less enzyme is required, for instance, and in the case of an inhibitor having a pharmacological or toxicological effect as a result of modifying an enzyme, the experimental conditions resemble what might occur in the intact animal.[23]

It is clear that phenylglyoxal irreversible inhihits GABA aminotransferase from *Pseudomonas fluorescens*. The rate of inactivation was proportional to the inhibitor concentration, thus no evidence of saturation kinetics was evident (Figure 5), indicating that no measurable reversible complexation between enzyme and inhibitor occurred. The inactivation rate was unaffected by changes in the concentration of either of the substrates, but increasing pyridoxal 5′-phosphate concentrations led to a gradual decrease in the apparent second order rate constant for inactivation. These observations suggest that phenylglyoxal is not modifying part of the enzyme to which GABA or α-ketoglutarate bind but is possibly modifying amino acid residues at the cofactor binding site. Since phenylglyoxal preferentially modifies arginyl residues,[6] such residues could be important for cofactor binding at the GABA aminotransferase active site. It has previously been demonstrated, in fact, that phenylglyoxal can inactivate mouse brain GABA aminotransferase and that pyridoxal 5′-phosphate can protect against this type of inhibition.[7]

Acknowledgements

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References

1. Tunnicliff, G. (1986) in *Neuromethods, Vol. 5 – Neurotransmitter Enzymes* (Boulton, A.A., Baker, G.B. and Yu, P.H., eds.) pp. 389–419. Humana Press, Clifton, NJ.
2. Krnjevic, K. (1991) in *GABA Mechanisms in Epilepsy* (Tunnicliff, G. and Raess, B.U., eds.) pp. 47–87. Wiley-Liss, NY.
3. Moses, U. and Churchich, J.E. (1980) *Biochim. Biophys. Acta*, **613**, 392–400.
4. Kim, D.S. and Churchich, J.E. (1981) *Biochem. Biophys. Res. Commun.*, **99**, 1333–1340.
5. Kim, J.T. and Churchich, J.E. (1991) *Biochim. Biophys. Acta*, **1077**, 187–191.
6. Takahashi, K. (1968) *J. Biol. Chem.*, **243**, 6171–6179.
7. Tunnicliff, G. (1980) *Biochem. Biophys. Res. Comm.*, **97**, 160–165.
8. Kwon, O., Park, J. and Churchich, J.E. (1992) *J. Biol. Chem.*, **267**, 7215–7216.
9. De Biase, D., Maras, B., Bossa, F., Barra, D. and John, R.A. (1992) *Eur. J. Biochem.*, **208**, 351–357.
10. Medina-Kauwe, L.K., Tillakaratne, N.J.K., Wu, J.-Y. and Tobin, A.J. (1994) *J. Neurochem.*, **62**, 1267–1275.
11. Richardson, I.B., Hurley, S.K. and Hynes, M.G. (1989) *Mol. Gen. Genet.*, **217**, 118–125.
12. Andre, B. and Jauniaux, J.-C. (1990) *Nucleic Acids Res.*, **18**, 3049.
13. Bartsch, K., von Johnn-Marteville, A. and Schulz, A. (1990) *J. Bacteriol.*, **172D**, 7035–7042.
14. Park, J., Osei Y.D. and Churchich, J.E. (1993) *J. Biol. Chem.*, **268**, 7636–7639.
15. Blessinger, K.J. and Tunnicliff, G. (1992) *Biochem. Cell Biol.*, **70**, 716–719.
16. Birchmeier, W. and Christen, P. (1974) *J. Biol. Chem.*, **249**, 6311–6315.
17. Ko, Y.H. and MaFadden, B.A. (1990) *Arch. Biochem. Biophys.*, **278**, 373–380.

18. Lowe, P.N. and Perham, R.N. (1984) *Biochemistry*, **23**, 91–97.
19. Okamoto, M. and Morino, Y. (1973) *J. Biol. Chem.*, **248**, 82–90.
20. Yonaha, K., Suzuki, K. and Toyama, S. (1985) *Eur. J. Biochem.*, **146**, 101–106.
21. Tsou, C.-L. (1988) *Adv. Enzymol.*, **61**, 381–436.
22. Tian, W.-X. and Tsou, C.-L. (1982) *Biochemistry*, **21**, 1028–1032.
23. Liu, W. and Tsou, C.-L. (1986) *Biochim. Biophys. Acta*, **870**, 185–190.