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Communication

Kinetics of inactivation of glutamate decarboxylase by cysteine-specific reagents

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Mercuric chloride, *p*-chloromercuribenzoate and 5,5′-dithiobis(2-nitrobenzoic acid) irreversibly inhibited the activity of *Escherichia coli* glutamate decarboxylase. Their second order rate constants for inactivation are 0.463 $\mu M^{-1}min^{-1}$, 0.034 $\mu M^{-1}min^{-1}$, 0.018 $\mu M^{-1}min^{-1}$, respectively. The characteristics of the inhibition by the three thiol-group reagents supports the idea that cysteinyl residues at the binding sites for the cofactor and/or the substrate are important for enzyme activity in *E. coli*.

In the vertebrate central nervous system γ-aminobutyric acid (GABA) is considered as the major inhibitory chemical transmitter [1]. This amino acid is synthesized from L-glutamic acid by the action of L-glutamate decarboxylase (GAD; EC 4.1.1.15) which requires pyridoxal 5′-phosphate (PLP) as cofactor [2]. GABA is also synthesized by plants and microorganisms, although in those organisms not possessing nerve tissue, the function of this amino acid is not quite clear. Evidence for *Escherichia coli* suggests that GAD can protect the pathogen from acid shock in the stomach [3].

A considerable number of inhibitors of mammalian GAD have been identified, including residue-specific reagents which have given clues about those amino acids that might be vital for catalytic activity [4, 5]. From such studies evidence has emerged that sulfhydryl groups of certain cysteinyl residues are important for enzyme activity [6, 7].

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Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoate); GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; pCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; PLP, pyridoxal 5′-phosphate.

Glutamate decarboxylase from microorganisms has not been studied as thoroughly as the mammalian enzyme. It is known, however, that the enzyme from *Streptococcus pneumoniae* has a molecular mass of 54 400 Da and exhibits 28% homology with GAD_{65} from brain [8]. GAD from *E. coli* and *Clostridium perfringens* consists of six identical subunits each with a molecular mass of about 50 000 Da [9, 10]. As in eukaryotes, the prokaryotic enzyme (*E. coli*) is encoded by two distinct genes whose sequences have been determined [11–13]. The protein products differ in only five amino-acid residues and their functional properties are identical. The enzyme from *E. coli* shows different physical and catalytic properties from the mammalian protein but does exhibit some similarities to the brain enzyme as far as its inhibition by substrate analogues and divalent cations, although quantitative differences do exist [14].

Compounds which react specifically with some amino acids can be useful for determination of the catalytic activity of enzymes. Loss of enzyme activity and protection against this loss by substrates or cofactors on exposure of enzyme to these reagents provides evidence that the respective amino acids are essential for biological activity. There is already some evidence that sulfhydryl groups are important for bacterial GAD activity [15–17]. With this in mind, the present investigation was undertaken to determine the kinetic mechanism of the action of several cysteine-specific reagents on GAD from *E. coli.*

MATERIALS AND METHODS

Enzyme and chemicals. L-Glutamate decarboxylase (40 units/mg protein) from *E. coli*, L-glutamic acid, pyridoxal 5′-phosphate, *N*-ethylmaleimide, *p*-chloromercuribenzoate, chloroacetamide, iodoacetate, 5,5′-dithiobis(2-nitrobenzoic acid) and mercuric chloride were obtained from Sigma Chemical Co. L-[1-^{14}C]Glutamate (54 mCi/mmol) was purchased from Research Products International.

Glutamate decarboxylase assay. The activity of the enzyme was measured by incubating 0.02 units at 37°C in the presence of 1 ml of 50 mM sodium acetate/acetic acid buffer, pH 4.6, containing 0.4 mM [^{14}C]glutamate, 0.05 mM PLP and 120 mM NaCl. The evolved radioactive CO_2 was trapped in 0.1 M potassium hydroxide. The reaction was terminated by the addition of 0.1 ml of 0.8 N H_2SO_4 and the incubation continued for a further 30 min to ensure the release of all carbon dioxide. Radioactivity was counted in a Beckman LS100 liquid-scintillation spectrometer. Flasks containing no enzyme were used as blanks.

Inactivation of glutamate decarboxylase. For the inactivation experiments, enzyme was incubated at room temperature in sodium acetate/acetic acid buffer, pH 4.6, with several different concentrations of inhibitor, with or without glutamate or PLP, for various lengths of time. In the course of the incubation, 20 μl aliquots of the mixture were successfully removed and added to 1.98 ml of acetate buffer containing 0.4 mM radioactive glutamate, 0.05 mM PLP and 120 mM NaCl. The activity of the enzyme was then measured by incubation at 37°C as described above.

RESULTS

The enzyme activity was assayed as described in Methods at pH 4.6 in the presence of DTNB, chloroacetamide, iodoacetate, NEM, pCMB or mercuric chloride. Only mercuric chloride, pCMB and DTNB inhibited enzyme activity (Table 1). In each case the inhibition was irreversible since, if enzyme before the assay was diluted 50-fold with buffer, the degree of inhibition was the same as if each inhibitor had been present during the incubation.

Under the conditions of the inactivation experiments, the rate of activity loss exhibited pseudo-first-order kinetics and was proportional to inhibitor concentration (Fig. 1). At

the highest inhibitor concentration (3 μM), however, pseudo-first-order kinetics was observed for the first 15 min only.

The observed rates of inactivation (k_{obs}), which are the pseudo-first-order rate constants, can be related to the inhibitor concentration [I] by the equation:

$$k_{obs} = k_2[I]^n$$

where n is the reaction order and k_2 is the second-order rate constant [18]. Therefore these results are consistent with the inactivation process following second-order kinetics as a bimolecular reaction:

$$E + I \xrightarrow{k_2} E\text{–}I$$

where E is enzyme, I is inhibitor, and E–I is the stable enzyme–inhibitor complex. The simple bimolecular rate constant (k_2) for $HgCl_2$ was calculated as 0.463 μM^{-1} min^{-1}. This was done by multiplying the slope of the lines by 2.303 and dividing by the inhibitor concentration. Further experiments were done under identical conditions and are summarized in the inset to Fig. 1. The lack of an intercept on the y axis supports the idea of a simple bimolecular reaction during which no measurable transient inhibitor–enzyme complex was formed; i.e., the mercuric chloride was not acting as an affinity label.

A similar pattern of inactivation was observed when either pCMB or DTNB was the inhibitor (Figs. 2 and 3). In both cases inactivation followed pseudo-first-order kinetics, although at the highest DTNB concentration (100 μM), pseudo-first-order kinetics was seen for the first 12 min only. As in the case with mercuric chloride, the increase in the rate of inactivation was proportional to the inhibitor concentration (Figs. 2 and 3, inset), indicating that no transient enzyme–inhibitor complex was formed. The second-order rate constant was calculated as 0.034 μM^{-1} min^{-1} and 0.018

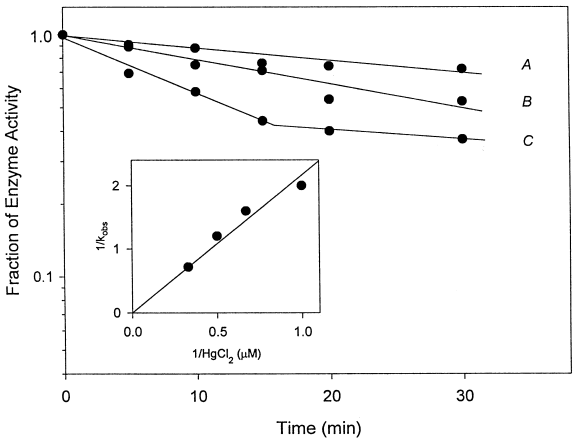

Figure 1. Remaining enzyme activity as a function of $HgCl_2$ concentration and time of exposure.

A = 1.5 μM $HgCl_2$ and 1 mM pyridoxal 5′-phosphate; *B* = 1.5 μM $HgCl_2$; *C* = 3.0 μM $HgCl_2$. In each case the rate of inactivation (k_{obs}) was obtained by multiplying the slope of the line by 2.303. Each point is the mean of four determinations and the coefficient of variation was never greater than 10%. The enzyme activity at 100% = 16.5 μmol/min per mg protein. The inset shows the reciprocal of k_{obs} plotted against the reciprocal of inhibitor concentration. For the sake of clarity, the data for additional two points in the inset are not shown in the main graph.

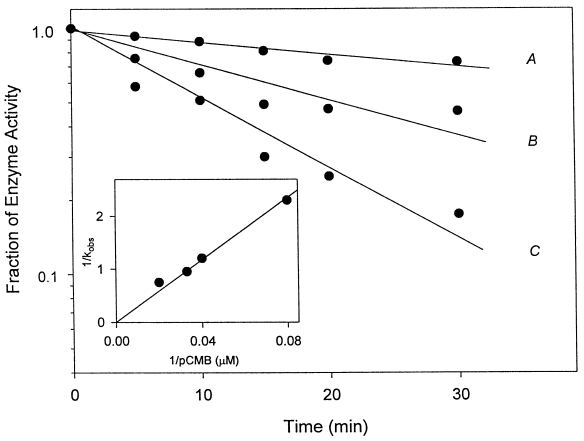

Figure 2. Remaining enzyme activity as a function of pCMB concentration and time of exposure.

A = 25 μM pCMB and 1 mM PLP; *B* = 25 μM pCMB; *C* = 50 μM pCMB. For other details see legend to Fig. 1.

$\mu M^{-1}min^{-1}$ for pCMB and DTNB, respectively.

If PLP was present during the exposure of enzyme to either of the three inhibitors, the rate of inactivationwas reduced in each case (Figs. 1, 2 and 3). The pro tec tion was con cen tra tion de pend ent, al though only one con cen tration is illustrated in the graphs. On the other hand, the presence of 10 mM glutamic acid reduced the rate of inactivation only by DTNB but not by the other two compounds.

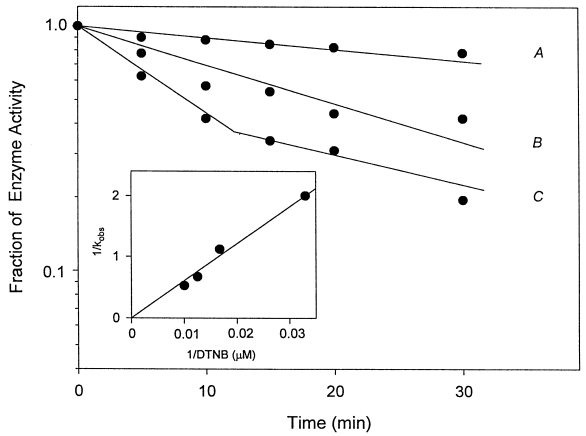

Figure 3. Remaining enzyme activity as a func tion of DTNB con cen tra tion and time of exposure.

A = 60 μM DTNB and 1 mM pyridoxal 5′-phos phate; *B* = 60 μM DTNB and 10 mM glu ta mate; *C* = 60 μM DTNB; *D* = 100 μM DTNB.

In one experiment the effects of increasing pH on the potential inhibitory action of iodoacetate, NEM or chloroacetamide was monitored. The enzyme was assayed at pH 4.0, 5.0, 6.0, 7.0 or 8.0 in the ab sence or pres ence of 100 μM of each com pound. In the ab sence of inhibitor, the enzyme activity reached a peak between pH 4.0 and 5.0. Iodoacetate had no marked ef fects on en zyme activity but both NEM and chloroacetamide showed a significant inhibitory effect at pH 6.0 and higher pH values (not shown).

DISCUSSION

Glutamate decarboxylase from *E. coli* was exposed to six different sulfhydryl-group reagents at pH 4.6, but the ac tiv ity was in hib ited by $HgCl_2$, pCMB and DTNB only. In each case, the inhibition was irreversible. The three inhibitors behaved similarly towards the enzyme. Each pro duced an in ac ti va tion that followed pseudo-first-order ki net ics and was linear with inhibitor concentration. Consequently, there was no evidence for the compounds producing readily reversible enzyme–inhibitor complexes and therefore no inhibitor binding constant could be cal cu lated. The other three sulfhydryl-selective reagents — iodoacetate, NEM and chloroacetamide—had no ap pre cia ble ef fect on en zyme activity. This might be explained by the relatively acidic con di tions of the re ac tion. The re ac tiv ity of these re agents is pH de pend ent, the

Ta ble 1. Ef fects of sulfhydryl-group re agents on *E. coli* glu ta mate de car box yl ase ac tiv ity at pH 4.6.

Reagent	Percentinhibition
$HgCl_2$	80.1 ± 11.0
p-Chloromercuribenzoate	68.5 ± 7.7
5,5′-Dithiobis(2-nitrobenzoate)	50.3 ± 11.1
Iodoacetate	4.6 ± 3.3
N-Ethylmaleimide	0.9 ± 6.3
Chloroacetamide	–0.3 ± 0.4

En zyme as sayed as de scribed in the Ma te rials and Methods. $HgCl_2$ con cen tra tion = 1 μM, all other com pounds = 100 μM. Values rep re sent mean (± S.D.) of 10 de ter mi na tions.

optimum range being pH 6–8. Indeed, when NEM or chloroacetamide were exposed to the enzyme at pH 6.0 or higher, an inhibition of enzyme activity was observed (not shown). This is in contrast to both mercuric chloride and pCMB whose reaction with proteins is the highest at about pH 5 [19].

The cofactor PLP was able to protect against the inactivation by the two mercurial compounds, as well as by DTNB. The substrate, however, did not protect against the inhibition by the mercurials but did protect against inhibition by DTNB. Consequently, these data provide evidence that essential cysteinyl residues reside at or near the cofactor binding site. In fact, previous studies had indicated that pCMB and mercuric chloride could adversely affect the activity of this enzyme although no kinetic data were provided [14, 16]. Actually, both Strausbauch & Fischer [9] and Fonda [15] have used DTNB to estimate the number of cysteinyl residues present in each subunit of the enzyme. The estimates were either 9 or 10 cysteines per subunit. Further, bromopyruvate was shown to inactivate *E. coli* GAD by alkylating one essential sulfhydryl group on each subunit [15]. In this regard, the bacterial enzyme resembles GAD from mammalian brain since the latter is susceptible to inhibition by reagents which react with thiol groups [6, 7, 20, 21].

In summary, we have shown that GAD from *E. coli* was irreversibly inhibited by the three sulfhydryl-group reagents, mercuric chloride, pCMB and DTNB, with mercuric chloride being by far the most effective. Since pyridoxal 5′-phosphate protected the enzyme against inhibition by all three compounds, it might be concluded that cysteinyl residue is important for cofactor function. Glutamate offered protection only against the effects of DTNB, suggesting cysteinyl residues also play a role in glutamate binding to the enzyme. However, it is not clear why it did not protect against the action of $HgCl_2$ and pCMB.

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