Inactivation of Glutamate Dehydrogenase and Glutamate Synthase from *Bacillus megaterium* by Phenylglyoxal, Butane-2,3-dione and Pyridoxal 5'-Phosphate

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Reaction of phenylglyoxal with glutamate dehydrogenase (EC 1.4.1.4), but not with glutamate synthase (EC 2.6.1.53), from *Bacillus megaterium* resulted in complete loss of enzyme activity. NADPH alone or together with 2-oxoglutarate provided substantial protection from inactivation by phenylglyoxal. Some 2 mol of [^{14}C]phenylglyoxal was incorporated/mol of subunit of glutamate dehydrogenase. Addition of 1 mM-NADPH decreased incorporation by 0.7 mol. The K_i for phenylglyoxal was 6.7 mM and K_s for competition with NADPH was 0.5 mM. Complete inactivation of glutamate dehydrogenase by butane-2,3-dione was estimated by extrapolation to result from the loss of 3 of the 19 arginine residues/subunit. NADPH, but not NADH, provided almost complete protection against inactivation. Butane-2,3-dione had only a slight inactivating effect on glutamate synthase. The data suggest that an essential arginine residue may be involved in the binding of NADPH to glutamate dehydrogenase. The enzymes were inactivated by pyridoxal 5'-phosphate and this inactivation increased 3–4-fold in the borate buffer. NADPH completely prevented inactivation by pyridoxal 5'-phosphate.

Inactivation of NADH-dependent bovine glutamate dehydrogenase by phenylglyoxal and butane-2,3-dione has been reported (David *et al.*, 1976; Pal & Colman, 1976). Four or five of the 30 arginine residues/subunit are modified in the course of complete inactivation in the absence of protective substrates. Partial protection against inactivation is provided by NAD^+ or 2-oxoglutarate when added separately. The most marked protection is provided by the combined addition of the coenzyme and substrate (Pal & Colman, 1976). Blumenthal & Smith (1975) have found that arginine residues of glutamate dehydrogenase from *Neurospora* play an important role in the binding of NADPH.

The present paper describes the presence of arginine residues (and possibly a lysine residue) of glutamate dehydrogenase from *Bacillus megaterium* that may interact with the inhibitors in the region of the active site.

Experimental

Materials

Glutamate synthase and glutamate dehydrogenase were purified from *Bacillus megaterium* as described by Hemmilä & Mäntsälä (1978). Phenylglyoxal and

butane-2,3-dione were purchased from Aldrich Chemical Co., Beerse, Belgium. [carbonyl- ^{14}C]-Acetophenone was obtained from New England Nuclear Corp., Boston, MA, U.S.A., and was used to prepare [^{14}C]phenylglyoxal by the method of Riley & Gray (1947). The specific radioactivity of the product was 0.11 mCi/mmol.

Inactivation experiments

Glutamate synthase and glutamate dehydrogenase were inactivated at 25°C in reaction mixtures containing 25 mM-potassium borate buffer, pH 7.6, phenylglyoxal or butane-2,3-dione and coenzyme and substrates as indicated in the Figure legends. The time course of inactivation was followed by removing samples (0.01 ml) at the intervals indicated and diluting 100-fold into the enzyme assay mixture. Activities were determined as described by Hemmilä & Mäntsälä (1978). The K_i for inactivation by phenylglyoxal was calculated by the method of Meloche (1967).

Determination of modified arginine residues

Glutamate dehydrogenase (0.86–1.29 mg) was modified by reaction with 5 mM-phenylglyoxal or butane-2,3-dione in 25 mM-borate buffer (pH 7.6) in a final volume of 0.8 ml. Incubation was at room temperature (25°C) for 5–90 min to obtain finally over 90% inactivation. The reaction was terminated

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid

by rapid gel filtration on a column (1 cm $\times$ 25 cm) of Sephadex G-25. The eluate was dialysed for 2 h against cold water and freeze-dried. The modified enzyme was hydrolysed *in vacuo* for 24 h at 110°C in 4 M-methanesulphonic acid containing 0.4% 3-(2-aminoethyl)-indole (Simpson *et al.*, 1976). The hydrolysate was analysed in a Perkin-Elmer KLA-5 amino acid analyser.

Results

Inactivation of glutamate synthase and glutamate dehydrogenase by phenylglyoxal

A two-step procedure was used to demonstrate inactivation. The enzyme and the inhibitor were incubated under appropriate conditions in the first

Fig. 1. Inactivation of glutamate dehydrogenase and glutamate synthase in the presence and absence of substrates and cofactors

The reaction rate was monitored by measuring the loss of enzymic activity and the data are shown in a semi-logarithmic representation. Except for the control all the reaction mixtures (0.1 ml) contained 25 mM-potassium borate buffer, pH 7.6, 0.34 mg of protein, 5 mM-phenylglyoxal and substrates as indicated. Samples (0.01 ml) were removed and assayed for glutamate dehydrogenase (a, b and c) and glutamate synthase (d). (a) Symbols: $\blacktriangle$, without phenylglyoxal; $\circ$, 5 mM-phenylglyoxal; $\bullet$, plus 20 mM-glutamate; $\square$, plus 1 mM-NADH; $\triangle$, plus 20 mM-glutamine. (b) Symbols: $\circ$, 5 mM-phenylglyoxal; $\bullet$, plus 100 mM- NH_4Cl ; $\triangle$, plus 20 mM-2-oxoglutarate. (c) Symbols: $\circ$, 5 mM-phenylglyoxal; $\bullet$, plus 1 mM-NADPH; $\triangle$, plus 1 mM-NADPH + 20 mM-2-oxoglutarate. (d) Symbols: $\circ$, 5 mM-phenylglyoxal; $\bullet$, plus 1 mM-NADPH + 20 mM-2-oxoglutarate.

step and then samples of enzyme were removed and diluted 100-fold into the assay mixture. Phenylglyoxal inactivated glutamate synthase by about 20% within 30 min in borate (Fig. 1d), Hepes or Tris buffers. However, when the enzyme was first treated with 50 μM -*p*-mercuribenzoate and then with 5 mM-phenylglyoxal, addition of 100 mM-2-mercaptoethanol almost completely restored the glutamine-dependent activity. As reported by Hemmilä & Mäntsälä (1978), 50 μM -*p*-mercuribenzoate inactivated glutamine-dependent glutamate synthase by nearly 90% within 5 min. Spectrophotometric titrations show that 90–100% inactivation of glutamine-dependent glutamate synthase from *Escherichia coli* is accompanied by incorporation of approximately 1.7 mol of *p*-mercuribenzoate/mol of enzyme protomer (Mäntsälä & Zalkin, 1976). Supposedly low concentrations of *p*-mercuribenzoate (50 μM) react only with the most reactive amino acid residues. This result suggests that there is no inactivation of glutamate synthase resulting from interaction of phenylglyoxal with an arginine residue (or residues), but that the inactivation by phenylglyoxal results from reaction with the residue that reacts with *p*-mercuribenzoate. Another possibility is that modification of a residue (or residues) by phenylglyoxal or *p*-mercuribenzoate precludes modification by the alternative reagent occurring in the same subunit.

Cyclohexane-1,2-dione (Blumenthal & Smith, 1975), butane-2,3-dione (Pal & Colman, 1976; David *et al.*, 1976) and phenylglyoxal (David *et al.*, 1976) have been reported to react with arginine residues of glutamate dehydrogenase from *Neurospora crassa* and bovine liver respectively. Fig. 1 shows the relative rates of inactivation by phenylglyoxal of glutamate dehydrogenase and glutamate synthase from *B. megaterium* with and without added substrates. As shown, 5 mM-phenylglyoxal inactivated glutamate dehydrogenase by about 80% within 30 min. The reaction followed pseudo-first-order kinetics until 90% of the enzyme had been inactivated and prolonged incubation (about 90 min) with phenylglyoxal resulted in complete inactivation. When the modification was performed in the presence of NADPH (1 mM) or NADPH (1 mM) + 2-oxoglutarate (20 mM) the rate of inactivation was significantly decreased (Fig. 1c). 2-Oxoglutarate alone (20 mM) decreased this rate of inactivation by about 20% (Fig. 1b). NADH, NH_4Cl , glutamine or especially glutamate had little effect (Figs. 1a and b).

The possible reactive thiol group was ruled out in the following procedure. The enzyme was first completely inactivated by 50 μM -*p*-mercuribenzoate and then incubated in the presence of 5 mM-phenylglyoxal. The addition of 100 mM-2-mercaptoethanol did not reactivate glutamate dehydrogenase, indicating that covalent binding of phenylglyoxal does not occur at the cysteine residue.

Determination of modified arginine residues

Two methods were used to determine the stoichiometry for binding of phenylglyoxal to glutamate dehydrogenase. In one method, the enzyme was incubated with phenylglyoxal at room temperature. The remaining activity and modification of arginine residues were assayed as described in the Experimental section and in the legend to Fig. 2. Fig. 2 shows a plot of the extrapolated percentage activity against the modified arginine residues. Extrapolation of the amount of phenylglyoxal required to completely inactivate the enzyme yielded the value 2.4 mol of phenylglyoxal/mol of subunit. In the other method, inactivation experiments were carried out as described above and the number of arginine residues modified was determined by incorporation of [14 C]phenylglyoxal into glutamate dehydrogenase. After incubation of glutamate dehydrogenase with [14 C]phenylglyoxal the reaction mixtures were freed from excess of [14 C]phenylglyoxal by gel filtration on a column (1 cm $\times$ 25 cm) of Sephadex G-25 as described in the legend to Table 1.

As shown in Fig. 1, inactivation by 5 mM-phenylglyoxal was linear with time up to 90% inactivation. The incorporation of phenylglyoxal, on the other hand, was linear with time up to 60% loss of activity had occurred (see also Fig. 2). The non-linear relationship of inactivation and incorporation indicates that the amount of inactivation is not directly proportional to the amount of [14 C]phenylglyoxal utilized. These results suggest that this incorporation involves residues that are not directly affecting the activity. Extrapolation of the amount of [14 C]phenylglyoxal directly required to inactivate glutamate dehydrogenase yields the value 1.9 mol of phenylglyoxal/mol of subunit. Addition of 1 mM-

Fig. 2. Determination of the number of phenylglyoxal-binding sites

The extrapolated remaining activity is plotted against molar ratio of phenylglyoxal/enzyme. The reaction mixture (0.8 ml) contained 1.06 mg of glutamate dehydrogenase and 5 mM-phenylglyoxal in 20 mM-borate buffer, pH 7.6. Incubation at 25°C was for 5–50 min. The reaction mixtures were filtered rapidly through a column (1 cm $\times$ 25 cm) of Sephadex dialysed for 2 h against cold water, freeze-dried and finally hydrolysed as described in the Experimental section.

NADPH to the reaction mixture decreased the incorporation of phenylglyoxal by 0.7 mol/mol of subunit. Combined addition of 1 mM-NADPH and 20 mM-2-oxoglutarate decreased incorporation by 0.8 mol/mol of subunit. NADH had little effect (Table 1).

Inactivation by butane-2,3-dione

Butane-2,3-dione (5 mM) inactivated glutamate dehydrogenase completely in the borate buffer (pH 7.6) within 35–40 min (Fig. 3). This inactivation was stimulated by, but does not require, borate. Inactivation was pseudo-first-order for more than 70% of the reaction. The most powerful protection was provided by the combined addition of NADPH (1 mM) and 2-oxoglutarate (20 mM) (Fig. 3). More than 2 arginine residues (19 arginine residues/subunit; Hemmälä & Mäntsälä, 1978) were modified during 90–100% inactivation of the enzyme by phenylglyoxal. Incorporation of butane-2,3-dione by the same method of modification was therefore examined (Fig. 4) and 2.8 mol of arginine residues/subunit was modified during this treatment.

Inactivation by pyridoxal 5'-phosphate

Kazarinoff & Snell (1976) have reported that pyridoxal 5'-phosphate protects serine dehydratase

Table 1. Effect of substrates on the incorporation of [14 C]phenylglyoxal into glutamate dehydrogenase

The reaction mixture (0.8 ml) contained 0.31 mg of glutamate dehydrogenase, 0.8 mM-phenylglyoxal (sp. radioactivity 0.04 mCi/mmol) and substrates as indicated. Samples (0.02 ml) were assayed for glutamate dehydrogenase activity, and after inactivation a sample (0.5 ml) was passed through a column (1 cm $\times$ 25 cm) of Sephadex G-25 and dialysed for 1 h against 50 mM-potassium phosphate buffer, pH 7.2.

Additions	Phenylglyoxal incorporation (mol/mol of subunit)
None	1.9
NADPH (1 mM)	1.2
NADPH (1 mM) + 2-oxoglutarate (20 mM)	1.1
2-Oxoglutarate (20 mM)	1.7
NADH (1 mM)	2.1

Fig. 3. Inactivation of glutamate dehydrogenase by butane-2,3-dione

Reaction mixtures contained 5 mM-butane-2,3-dione. Otherwise the experimental conditions were the same as in the legend to Fig. 1. Symbols: ○, 5 mM-butane-dione; △, + 1 mM-NADPH; □, + 1 mM-NADPH + 20 mM-2-oxoglutarate; ●, + 1 mM-NADH.

Fig. 4. Correlation of inactivation of glutamate dehydrogenase with modification of arginine residues by butane-2,3-dione

Incubation was for 5–30 min at room temperature. Otherwise the experimental conditions were the same as in the legend to Fig. 2.

against inactivation by butane-2,3-dione. Pyridoxal 5'-phosphate, on the other hand, inactivates bovine liver glutamate dehydrogenase (Chen & Engel, 1975). Pyridoxal 5'-phosphate resulted in a biphasic loss of glutamate dehydrogenase from *B. megaterium*

(Fig. 5). Borate buffer increased the effect of inactivation by about 3–4-fold compared with the effect in Hepes buffer. In the absence of pyridoxal 5'-phosphate, glutamate dehydrogenase was stable under the conditions of modification. NADPH (2 mM) and 2-oxoglutarate (60 mM) completely prevented inactivation (Fig. 5a). About 80% reactivation of the inactive dehydrogenase was obtained by treatment with NADPH. One explanation favours a two-step mechanism for the reaction of glutamate dehydrogenase with pyridoxal 5'-phosphate. The first step is reversible formation of a non-covalent complex, which can then react further to form a Schiff base. The first-order covalent reaction occurs slowly and only 20% of initial activity was lost during 15 min inactivation (Fig. 5a). At 3 mM-pyridoxal 5'-phosphate glutamine-dependent glutamate synthase was also inactivated by about 50% within 10 min, and this inactivation was not prevented by 2-oxoglutarate (Fig. 5b). Alternatively interaction between borate and nicotinamide nucleotides could cause apparent protection. Interaction between borate and nicotinamide nucleotides or flavin-requiring enzymes have

Fig. 5. Inactivation of glutamate dehydrogenase and glutamate synthase by pyridoxal 5'-phosphate

The reaction mixtures (0.1 ml) contained 25 mM-potassium borate buffer, pH 7.6, 0.34 mg of protein, 2 mM-pyridoxal 5'-phosphate, and substrates as indicated. Samples (0.01 ml) were assayed for glutamate dehydrogenase (a) and glutamate synthase (b) activity. (a) Symbols: ○, 2 mM-pyridoxal 5'-phosphate; ●, 2 mM-pyridoxal 5'-phosphate + 2 mM-NADPH + 60 mM-2-oxoglutarate. The arrow shows the addition of 2 mM-NADPH. (b) Symbols: △, 3 mM-pyridoxal 5'-phosphate; ▲, 3 mM-pyridoxal 5'-phosphate + 60 mM-2-oxoglutarate.

Fig. 6. Kinetics for inactivation of glutamate dehydrogenase by phenylglyoxal

Assay conditions were the same as those described in the legend to Fig. 1. NADPH concentrations were 0 (○) and 1 mM (●).

been reported (Smith & Johnson, 1976; Roush & Norris, 1950). Borate alone, however, had no inactivating effect.

Kinetics of inactivation by phenylglyoxal and pyridoxal 5'-phosphate

The inactivation of glutamate dehydrogenase by phenylglyoxal exhibits saturation kinetics (Fig. 6). The inactivation constant (K_i) for phenylglyoxal was determined by using the method of Meloche (1967). When the inactivation half-time (τ) was plotted on the ordinate, competitive kinetics of protection by NADPH were observed (Fig. 6). K_i was 6.7 mM and the K_i for competition by NADPH was 0.5 mM. Although the K_m for NADPH was 8.7 μ M, the results suggest that both NADPH and phenylglyoxal compete for the same site on the enzyme.

Discussion

The almost complete protection against inactivation of glutamate dehydrogenase by phenylglyoxal or butane-2,3-dione afforded to the enzyme by NADPH or NADPH + 2-oxoglutarate is a very strong indication that the location of an essential arginine residue is in the region of the active site. NADH supports 2.3% of NADPH activity with glutamate dehydrogenase (Hemmilä & Mäntsälä, 1978). Similarly NADH protects only to a small extent from the interaction with phenylglyoxal or butane-2,3-dione. These results favour the conclusion that the positively charged guanidinium group of functional arginine interacts only with the negatively charged phosphate group of NADPH.

In the case of *Neurospora* glutamate dehydrogenase NADPH or 2-oxoglutarate did not protect against inactivation by cyclohexane-1,2-dione (Blumenthal & Smith, 1975). However, indirect evidence suggested that a modified arginine residue is located at or near the binding site for NADP⁺. The isolation of the peptide containing the modified arginine residue provided confirmation that the binding site is located at the active site (Austen & Smith, 1976). Direct evidence for the suggestion that an arginine residue is involved in coenzyme binding was provided by Lange *et al.* (1974), who found that phenylglyoxal-modified horse liver alcohol dehydrogenase no longer bound NADH. However, the possibility that the binding of NADPH to the enzyme causes a conformational change and makes the enzyme inaccessible to phenylglyoxal is not excluded. On the other hand, this effect would be a unique property of NADPH, since the other substrates were much less effective. Because a number of reports have suggested that arginine or lysine residues in proteins often play a role in the binding of negatively charged cofactors and substrates, we studied the effect of pyridoxal 5'-phosphate on the activities. Pyridoxal 5'-phosphate is not an effector of glutamate synthase or glutamate dehydrogenase, but inactivated both enzymes, suggesting that perhaps a lysine residue is located at, or close to, the active site. The substantial protection provided by NADPH suggests that pyridoxal 5'-phosphate competes with NADPH at the same binding site. Phenylglyoxal modifies the arginine residue at the binding site, whereas the inactivation caused by pyridoxal 5'-phosphate is partially reversible and can be removed by the addition of NADPH. The fact that the kinetics of inactivation of glutamate dehydrogenase by phenylglyoxal are first-order with a single rate constant is consistent with there being a single arginine (and possibly a lysine) residue whose blockage is responsible for inactivation.

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