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The Asymmetric Distribution of Enzymic Activity between the Six Subunits of Bovine Liver Glutamate Dehydrogenase

USE OF D- AND L-GLUTAMYL α-CHLOROMETHYL KETONES (4-AMINO-6-CHLORO-5-OXOHEXANOIC ACID)

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A method for the preparation of D- and L-glutamyl α-chloromethyl ketones (4-amino-6-chloro-5-oxohexanoic acid) is described. These chloromethyl ketones irreversibly inactivated bovine glutamate dehydrogenase, whereas several other related compounds had no adverse effect on the activity of the enzyme. The inactivation process was shown to be due to the modification of lysine-126. The time-courses for the inactivation and the incorporation of radioactivity from tritiated L-glutamyl α-chloromethyl ketone into the glutamate dehydrogenase were biphasic. The results were interpreted to suggest the involvement of 'negative co-operative' interactions in the reactivity of lysine-126. From the cumulative evidence it is argued that the first subunit of the enzyme, which takes part in catalysis, makes the largest, and the last the smallest, contribution to the overall catalysis. It is emphasized that three of the six subunits of the enzyme may possess as much as 80% of the total activity of bovine glutamate dehydrogenase.

The use of active-site-directed inhibitors in the last decade has made an invaluable contribution to our knowledge of the chemistry of the active-site regions of enzymes and has also laid the foundation for a rational design of compounds of potential therapeutic importance. The most widely quoted first man-made active-site-directed inhibitors were *N*-protected derivatives of lysine and phenylalanine in which the carboxyl group was replaced by a haloketone moiety (Baker, 1967; Shaw, 1970). Such compounds inhibited the action of trypsin and chymotrypsin, which normally hydrolyse *N*-protected peptide substrates. There are, however, a large number of other enzymes that act on amino acids and have a compulsory requirement for the availability of amino groups of the substrates. Therefore potential inhibitors of this class of enzymes are likely to be compounds that have a free amino group as well as an electrophilic centre, as shown in Scheme 1 [structures (IV) and (V)].

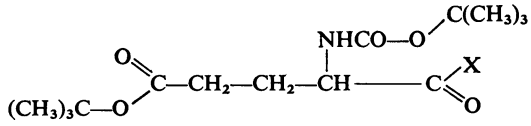
We have developed (Birch *et al.*, 1972) a method that under mild conditions allowed an efficient synthesis of chloromethyl ketone analogues of neutral amino acids. The method has subsequently been extended to the preparation of the chloromethyl ketone derivative of the basic amino acid lysine (Coggins *et al.*, 1974). In the present paper we report the preparation of the α-chloromethyl ketone derivatives of D- and L-glutamate [see structure (IV), Scheme 1] and describe their usefulness for exploring the chemistry of bovine glutamate dehydrogenase.

Experimental

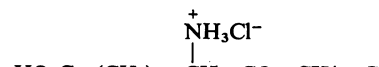
Materials

Glutamate dehydrogenase from bovine liver, NAD^+ and NADH were from Boehringer Corp. (London), Lewes, East Sussex BN7 1LG, U.K.; α-oxoglutarate, trypsin and *S*-carboxymethylcysteine were from Sigma Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.; 1-carboxymethylhistidine and 3-carboxymethylhistidine were from Calbiochem, Hereford, U.K.; all other chemicals were from BDH, Poole, Dorset, U.K. Butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] and NE250 were from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and Nuclear Enterprises, Sighthill, Edinburgh, Scotland, U.K., respectively.

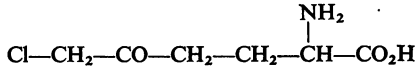
N^6-Carboxymethyl-lysine was synthesized by the method of Gundlach *et al.* (1959) and was purified by high-voltage paper electrophoresis in pH 3.1 buffer (pyridine/acetic acid/water, 3:52:130, by vol.). 3-Amino-1-chlorobutan-2-one (alanyl chloromethyl ketone; Ala-CH_2Cl) was prepared by the method of



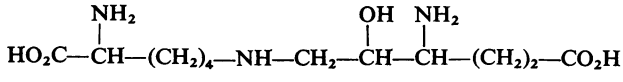
(I) X = —OH
(II) X = —O—CO—OC_2H_5
(III) X = —$\bar{C}$H—$\overset{+}{N}$≡N



(IVa) L Isomer
(IVb) D Isomer
(IVc) L Isomer (H* = ^{3}H)



(V)



(VI)

Gly-Gly-Ala-Lys*-Ala-Gly-Val-Lys
123 126 130

(VII)

Scheme 1. *Structures of the synthetic intermediates (I)–(V), the modified amino acid (VI) and the active-site peptide (VII)*

Lys* refers to the modified lysine residue.

Birch *et al.* (1972) and 2-amino-6-chloro-5-oxohexanoic acid [L-glutamyl γ-chloromethyl ketone; L-Glu(γ-CH_2Cl)] by the treatment of the corresponding diazoketone (Weygand *et al.*, 1958) with HCl. Throughout phosphate buffers refer to 0.05M-sodium phosphate.

Preparation of γ-t-butyl α-ethyl N-benzyloxycarbonylglutamate

In a 250ml round-bottom Quickfit flask fitted with a solid-CO_2 trap a solution of α-ethyl *N*-benzyloxycarbonylglutamate (10.0g) (Le Quesne & Young, 1950; Weygand & Hunger, 1962) in dichloromethane (60.0ml) with conc. H_2SO_4 (0.5ml) was added. 2-Methylpropene gas was passed through the solution until saturation. The flask was well stoppered after the removal of the trap and left for 3 days at room temperature (20°C). The flask was then cooled (−40°C) and carefully opened. The excess of 2-methylpropene was then allowed to evaporate and the reaction mixture was poured into a dilute solution (5%, w/v) of Na_2CO_3 (500ml). The dichloromethane layer was washed with water, dried (over Na_2SO_4), filtered and evaporated to dryness to give an oil (about 10.0g).

Preparation of γ-t-butyl N-benzyloxycarbonylglutamate

γ-t-Butyl α-ethyl *N*-benzyloxyglutamate (2.2g) was dissolved in acetone (18.0ml) and cooled to 5°C. A solution of 1M-NaOH (6.6ml) was added dropwise with shaking. After the addition was complete the reaction mixture was left at room temperature for 1h. The mixture was then evaporated to dryness under vacuum and the residue dissolved in water (25ml) and extracted with diethyl ether (2×25ml). The aqueous layer was acidified with dilute HCl to pH2 with cooling and the product extracted with ethyl acetate (2×50ml). The combined organic extract was washed with water (50ml), dried (over Na_2SO_4), filtered and the filtrate evaporated to dryness in vacuum to give an oil (2.0g).

Preparation of γ-t-butyl glutamate

γ-t-Butyl *N*-benzyloxycarbonylglutamate (2.2g) was dissolved in methanol (37.0ml); 10% (w/w)

Pd/charcoal (0.26g) was added and the flask was then mounted to an atmospheric hydrogenator. The hydrogenation was carried out for 4h, when the H_2 uptake ceased. The catalyst was then removed by filtration, and the filtrate evaporated to dryness in vacuum. The residue was crystallized from methanol/diethyl ether (yield, 1.2g). The D isomer had m.p. 182°C (Found: C, 53.1; H, 8.3; N, 6.6. Calc. for $C_9H_{17}NO_4$: C, 53.2; H, 8.4; N, 6.9%), and the L isomer had m.p. 184°C (Found: C, 53.2; H, 8.4; N, 6.8. Calc. for $C_9H_{17}NO_4$: C, 53.2; H, 8.4; N, 6.9%).

Preparation of γ-t-butyl N-t-butoxycarbonylglutamate (I)

A mixture of γ-t-butyl glutamate (1.0g), t-butyl 2,4,5-trichlorophenyl carbonate (2.5g), triethylamine (2.4ml), water (5.4ml) and 2-methylpropan-2-ol (8.2ml) was stirred at 60–70°C overnight. Most of the 2-methylpropan-2-ol was then removed under vacuum. After the addition of water the reaction mixture was acidified to pH3 at 0°C with a saturated solution of citric acid and the oily emulsion extracted with ethyl acetate (3×25ml). The combined organic extracts were washed with a saturated solution of $NaHCO_3$ (3×25ml) and the washings were acidified to pH3 with citric acid at 0°C. The latter solution was extracted with ethyl acetate (3×25ml). The combined organic extract was dried (over Na_2SO_4) and evaporated to dryness to give an oil (1.5g), which solidified at 0°C.

Preparation of γ-t-butyl N-t-butoxycarbonyl-1-diazomethylglutamate (III)

A mixture of γ-t-butyl *N*-t-butoxycarbonylglutamate (1.2g) and dry triethylamine (0.56ml) in dry diethyl ether (82ml) (freshly distilled over $LiAlH_4$) was cooled to −10°C and treated with ethyl chloroformate (0.46g). After 15min the precipitate of triethylamine hydrochloride was removed by filtration and the filtrate at once treated with an excess of dry ethereal diazomethane (150ml). After 30min at 0°C the excess of diazomethane was removed under vacuum at 30°C and the diethyl ether solution was washed with water, dried (over Na_2SO_4) and evaporated to dryness in a vacuum at 30°C. The residue, which solidified at 0°C, was found to be homogeneous by t.l.c. analysis. The i.r. spectra of the D and L isomers were identical and had $\nu_{max.}$ 1620 and 2200cm^{-1} for the diazoketone group (D isomer; Found: C, 54.8; H, 7.3; N, 11.6. L isomer; Found: C, 55.0; H, 7.5; N, 11.4. Calc. for $C_{15}H_{25}N_3O_5$: C, 55.0; H, 7.7; N, 12.8%).

Preparation of glutamyl α-chloromethyl ketone (IV)

A solution of γ-t-butyl *N*-t-butoxycarbonyl-1-diazomethylglutamate (0.1g) in Na-dried diethyl ether (2.0ml) at 0°C was saturated with dry HCl gas and the mixture was left at 0°C overnight. The precipitated chloromethyl ketone hydrochloride was filtered, washed with dry diethyl ether and kept over P_2O_5. The D-chloromethyl ketone had m.p. 128°C (decomp.) (Found: C, 33.7; H, 5.2; N, 6.3. Calc. for $C_6H_{11}Cl_2NO_3$: C, 33.3; H, 5.1; N, 6.5%). The L-chloromethyl ketone was hygroscopic and had m.p. 130°C (decomp.) (Found: C, 31.0; H, 5.3; N, 6.2. Calc. for $C_6H_{11}Cl_2NO_3$: C, 33.3; H, 5.1; N, 6.5%). The i.r. spectra of both the isomers were identical and in the carbonyl region had $\nu_{max.}$ 1700 and 1730cm^{-1}.

The tritiated analogues of the chloromethyl ketones were prepared similarly except that in these cases the D- or L-diazoketone derivative (100mg) was added to a solution of diethyl ether saturated at 0°C with dry HCl gas (2ml), which had previously been shaken with 20μl of ^3H_2O (5Ci/ml) to generate ^3HCl *in situ*. The yields of the chloromethyl ketone hydrochlorides (IV*c*) were 25–30mg. Since the samples obtained by this method should also contain radioactivity at the exchangeable amino hydrogen atoms the specific radioactivities of the chloromethyl ketones were determined as follows.

A weighed amount of the chloromethyl ketone was reduced with $NaBH_4$ in methanol at 4°C for 30min to stabilize the radioactivity. The methanol was removed under reduced pressure, 2ml of methanol was added and again evaporated. This was repeated five or six times. The residue was dissolved in a known volume of methanol and a portion was removed for radioactivity measurement. The specific radioactivity was different in different preparations and has been mentioned where necessary.

Determination of mol of inhibitor incorporated per mol of polypeptide chain of glutamate dehydrogenase

To determine the relationship between the incorporation of inhibitor (L-Glu-CH_2Cl) into protein and enzyme activity, glutamate dehydrogenase (2.5mg) was incubated with 6.6mM-L-[^{3}H]Glu-CH_2Cl (specific radioactivity 1.4×10^6c.p.m./μmol) in 0.05M-phosphate buffer, pH6.2 (2ml), at 30°C. A portion (50μl) was removed at various times (20, 40, 60, 120 and 180min) and assayed for enzyme activity as described below. At the same time another sample (0.3ml, 0.375mg of protein) was removed and precipitated with 10% (w/v) trichloroacetic acid. It was washed with 10% trichloroacetic acid (6×2ml) and dissolved in 1M-Hyamine hydroxide in methanol (0.5ml) by warming at 65°C for 15min. A sample was removed to determine the concentration of protein and the remaining solution was taken up in methanol (2ml) and radioactivity was determined as described under 'Radioactivity measurement' by using butyl-PBD.

The number of mol of the inhibitor incorporated into the enzyme was calculated on the basis of specific radioactivity of inhibitor and concentration of protein, assuming that glutamate dehydrogenase consists

of six subunits each with mol.wt. 50000 (Appella & Tomkins, 1966).

In another experiment, the enzyme (2.5mg) was incubated with 6.6mM-L-[^{3}H]Glu-CH_2Cl as described above. The enzyme activity was measured at various times (20, 40, 60, 120 and 180min). At the same time, a sample (0.3ml; 0.375mg of protein) was removed, reduced with $NaBH_4$ to stabilize the radioactivity and precipitated with 10% trichloroacetic acid (2ml). It was washed with 10% trichloroacetic acid (6×2ml) and dissolved in 1M-Hyamine hydroxide (0.5ml). Protein concentration and radioactivity were measured as described above. The number of mol of the inhibitor incorporated into the enzyme was determined as described above.

Protein determination

For the calculation of the number of mol of the inhibitor incorporated per mol of the polypeptide chain, the protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. It was established that standard curves constructed with either bovine serum albumin or authentic glutamate dehydrogenase were identical. The concentration of glutamate dehydrogenase for the assay was determined spectrophotometrically by using a value of 0.97 E_{280} unit for 1mg of enzyme/ml solution in a 1cm-path-length cell (Olson & Anfinsen, 1952). We are most grateful to Dr. David Bloxham for the experiment establishing that bovine serum albumin is an appropriate standard for the determination of glutamate dehydrogenase. The protein in column eluates was determined by u.v. absorption at 280nm.

Radioactivity measurement

Radioactivity of all samples were measured with an intertechnique scintillation counter; 10ml of scintillation fluid containing 0.8% butyl-PBD in toluene was used, but for aqueous samples NE250 fluid (Nuclear Enterprises) (1:10, v/v) was used.

Identification of the amino acid residue of glutamate dehydrogenase modified by L-Glu-CH_2Cl

The enzyme (20mg) was incubated with 6mM-L-[^{3}H]Glu-CH_2Cl (specific radioactivity 1.4×10^6 c.p.m./μmol) in 0.05M-phosphate buffer, pH6.2, at 30°C for 3h. The 92%-inactivated enzyme was extensively dialysed against water (4×2 litres) overnight at 4°C. The dialysed protein (3ml) was precipitated with 10% trichloroacetic acid (2ml), washed with diethyl ether (2×3ml) and dried by flushing with N_2 gas.

The precipitated labelled protein was subjected to performic acid oxidation by the method of Hirs (1956) and the resulting material was heated in 6M-HCl (2ml) at 110°C for 18h in an evacuated sealed tube. The acid hydrolysate was subjected to high-voltage paper electrophoresis in pH3.1 buffer (pyridine/acetic acid/water, 3:52:130, by vol.) along with standard carboxymethyl derivatives of N^6-lysine, N^1-histidine, N^3-histidine and cysteine.

Preparation of radioactive peptide

The enzyme (40mg) was incubated with L-[^{3}H]Glu-CH_2Cl (specific radioactivity 1.4×10^6 c.p.m./μmol) in 0.05M-phosphate buffer, pH6.2, at 30°C, and after 95% inactivation the labelled protein was reduced with $NaBH_4$ to stabilize the radioactivity. After extensive dialysis against water (5×2 litres) for 48h at 4°C, the protein was precipitated with 10% trichloroacetic acid, washed with 10% trichloroacetic acid (6×2ml), then with diethyl ether (2×2ml), and finally dried by flushing with N_2 gas. The precipitated protein was suspended in 0.2M-$NaHCO_3$ (6ml), transferred into a sonication vial (150ml) and sonically disrupted in a 100W MSE ultrasonic disintegrator at 20kHz. It was then incubated with trypsin (3mg in 0.2ml of 0.2M-$NaHCO_3$) at 33°C. After 15min the turbidity decreased, but digestion was continued for 11h, the pH being kept between 8 and 9 with 0.2M-$NaHCO_3$. The trypsin was then precipitated (by heating the incubation mixture in a boiling-water bath) and removed by centrifugation at 1000*g*. The soluble peptides, containing 90% of the radioactivity originally present in the modified protein, were applied to a column (1.5cm×150cm) of Sephadex G-25 (fine grade), which had been previously equilibrated in 10mM-NH_4HCO_3. The column was eluted with 10mM-NH_4HCO_3 and the fractions (3ml) were assayed for protein and radioactivity.

Determination of molar ratio of amino acids of labelled peptide

A sample of radioactive peptide (15nmol) was hydrolysed with 6M-HCl (50μl) in an evacuated sealed tube at 110°C for 18h. HCl was removed under reduced pressure in a vacuum desiccator at room temperature. The residue was dissolved in 0.01M-HCl (5ml) and analysed on a JEOL 6AH automatic amino acid analyser. A control sample was set up that contained an appropriate volume of eluate from Whatman 3MM paper to calculate the zero values.

In some of our amino acid-analysis experiments the lysine peak was not separated clearly from the ammonia peak and was integrated manually and the amount of lysine was calculated on the basis of an internal standard (norleucine) peak, which was also integrated manually. Validity of the method was tested by comparing the area of the peak for other amino acids integrated manually with the peak area for the same amino acids integrated by the integrator.

Modified lysine residue was eluted from the column of the amino acid analyser at the same time as unmodified lysine. To determine the amount of modified lysine, fractions from the column of the amino acid

analyser were assayed for radioactivity. The number of nmol of the modified lysine was calculated on the basis of the specific radioactivity of the reagent.

Results

Preparation of α-chloromethyl ketone analogues of D- *and* L-*glutamic acid*

γ-t-Butyl L-glutamate was converted into γ-t-butyl *N*-t-butoxycarbonyl-L-glutamate [Scheme 1; (I)], which was then treated with ethyl chloroformate to produce the mixed anhydride (II). The reaction of the latter with diazomethane gave the diazoketone (III), which was treated at 0°C with dry HCl gas in diethyl ether to give in 45% overall yield the hydrochloride of L-glutamyl α-chloromethyl ketone (IV*a*) ($[\alpha]_D$ +31°). When this operation was performed with ^{3}HCl gas, the labelled material (IV*c*) was obtained, which should, because of the method of preparation, contain ^{3}H at the chloromethyl group. The corresponding α-chloromethyl ketone in the D series (IV*b*) ($[\alpha]_D$ −30°) was produced analogously. The D- and L-chloroketone derivatives (IV*a* and IV*b*) had equal and opposite rotations at 365, 436, 546, 578 and 589nm (Fig. 1), thus proving that under the conditions of the synthesis the sensitive chiral centre was undisturbed. The fact that an aqueous solution of (IV*a*) or (IV*b*) underwent complete racemization at room temperature in 24h suggests that other methods (Thompson & Blout, 1973; Powers & Tuhy, 1973; Kurachi *et al.*, 1973) for the synthesis of chloromethyl ketones in which protecting groups are removed by treatment with aqueous strong mineral acids are unlikely to produce optically pure derivatives.

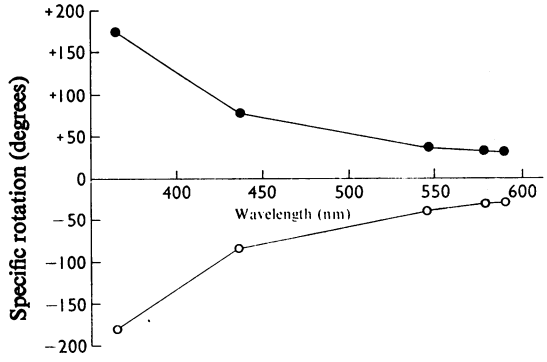


Fig. 1. *Specific optical rotations of* L (●) *and* D (○) *isomers of Glu-CH$_2$Cl at various wavelengths*

The optical rotations were determined in aqueous solutions (2.5mg of the compound/ml of water) with a 10cm-pathlength cell by using a Perkin–Elmer 141 polarimeter.

*Time-course and specificity of the inactivation of glutamate dehydrogenase by glutamyl α-chloromethyl ketone (Glu-CH$_2$Cl) (IV*a*)*

Bovine liver glutamate dehydrogenase consists of six identical subunits and catalyses the reaction (Appella & Tomkins, 1966):

$$HO_2C{-}CH_2{-}CH_2{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}CO_2H + NAD^+ \rightleftharpoons NADH + NH_3 + HO_2C{-}CH_2{-}CH_2{-}CO{-}CO_2H + H^+$$

Incubation of the enzyme at 30°C with the L-Glu-CH$_2$Cl (IV*a*) resulted in a time-dependent loss of the enzyme activity (Fig. 2). The dialysis of 95%-inactivated enzyme against 0.05M-sodium phosphate buffer for 18h at 4°C failed to produce any recovery of the activity, thus showing that the inactivation was due to an irreversible chemical modification. The time-course of inactivation was unexpected; 50% of the enzymic activity of glutamate was lost in about 20min, whereas the enzyme was about 90% inactivated only after 120min (Fig. 2). In the range 0.25–10.0mg of glutamate dehydrogenase/ml, the inactivation profile was independent of concentration. A similar biphasic inactivation pattern was also obtained when the enzyme was assayed by using the NADP–glutamate system. Several reasons for the observed inactivation pattern were possible. For example, it could be argued that the biphasic inactivation process was due to the decomposition of the inhibitor. To evaluate this possibility two types of experiment were designed. First, when the enzyme was incubated with the inhibitor, which had been preincubated with phosphate buffer at pH6.2 at 30°C for 30min, the biphasic inactivation process identical with that described above was obtained, suggesting that the unusual pattern of inactivation was not due to the decomposition of the inhibitor. Secondly, the enzyme was incubated with L-Glu-CH$_2$Cl as usual, and after 50% inactivation it was dialysed extensively against 0.05M-phosphate buffer. The 50%-inactivated enzyme was again incubated with the inhibitor under conditions similar to those described above, and it was found that the 50%-inactivated enzyme did not show a biphasic inactivation, but followed a slower 'first-order' reaction

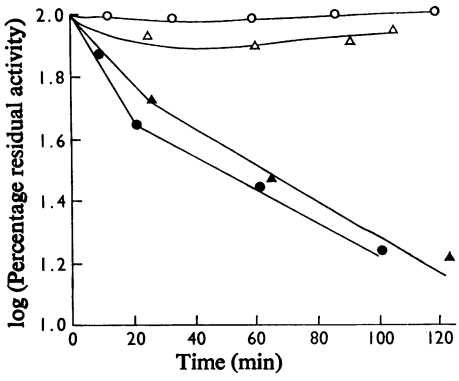


Fig. 2. *Inactivation of glutamate dehydrogenase by* L-*glutamyl α-chloromethyl ketone* (L-*Glu-CH_2Cl*) *and related compounds*

The enzyme (0.25mg/ml) was incubated in 0.05M-sodium phosphate buffer, pH6.2, at 30°C in the presence of: 6.0mM-L-Glu-CH_2Cl (●), 6.0mM-D-Glu-CH_2Cl (△); no addition (○); 6.0mM-L-Glu(γ-CH_2Cl) (○), 6.0mM-L-Glu-CH_2Cl+40mM-L-glutamate (▲); 6.0mM-Ala-CH_2Cl (▲); 6.0mM-chloropyruvate (▲). Samples (50μl) were removed at the times indicated and assayed as follows. The assay mixture (3.0ml), containing 0.1ml of 1M-NH_4Cl, 0.1ml of 0.1M-α-oxoglutarate (pH7.0), 0.1ml of NADH (2mg/ml), 2.7ml of 0.05M-sodium phosphate buffer, pH7.6, was equilibrated at 30°C and reaction was started by the addition of a portion (50μl) from the enzyme/inhibitor preincubated mixture. The reaction was monitored at 340nm. The results are expressed as the logarithm of percentage of activity remaining.

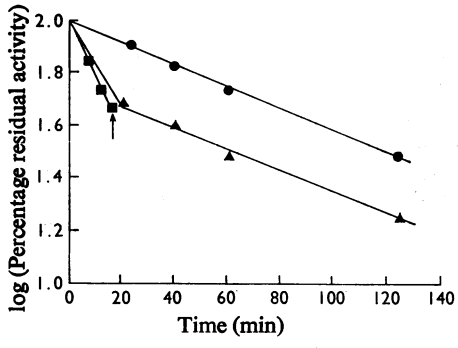


Fig. 3. '*First-order*' *inactivation of 50%-inhibited glutamate dehydrogenase by* L-*Glu-CH_2Cl*

The enzyme (0.25mg/ml) was incubated with L-Glu-CH_2Cl (6.0mM) in 0.05M-sodium phosphate buffer, pH6.2, at 30°C. After 50% inactivation (■), indicated by an arrow, the enzyme was dialysed against 0.05M-sodium phosphate buffer, pH6.2, at 4°C and again incubated with L-Glu-CH_2Cl under the same conditions (●). In a control experiment, the enzyme without preincubation was incubated with L-Glu-CH_2Cl (▲). Samples (100μl) were removed at the times indicated and assayed as described in Fig. 2. The results are expressed as the logarithm of percentage of residual enzyme activity in each case.

corresponding to the second phase normally observed by using the continuous inactivation method (Fig. 3).

The enzyme was completely protected against inactivation in the presence of L-glutamate (Fig. 2). This observation highlighted two facets: first, that the inactivation may involve the modification of a group at or near the active site, and secondly, that glutamate is able to bind to the enzyme in the absence of the coenzyme. The specificity of the inactivation was established by the fact that other chloroketones such as chloropyruvate, DL-alanyl chloromethyl ketone and particularly L-glutamyl γ-chloromethyl ketone (V) had no adverse effect on the activity of the enzyme. The alkylation reaction, however, did not show stereospecificity, since the D-glutamyl α-chloromethyl ketone inactivated the enzyme in a fashion similar to that noted with the L isomer (Fig. 2).

Time-course and stoicheiometry of incorporation of radioactivity from L-[^3H]*Glu-CH_2Cl into glutamate dehydrogenase*

Time-course studies on glutamate dehydrogenase inactivation by ^3H-labelled glutamyl α-chloromethyl ketone (IV*c*) and incorporation of radioactivity into the protein showed that both phenomena were biphasic and the 50%-inactivated enzyme had incorporated about 1mol of the inhibitor per mol of the hexamer. After the incorporation of about 3.8mol of the inhibitor per mol of the hexamer the enzyme was 96% inactivated (Fig. 4). The implication of these results, suggesting that the modification of about one-half of the subunits of the enzyme resulted in the loss of nearly all the activity, called for the design of several types of control experiments. The possibility was considered that the inhibitor containing radioactivity at the potentially labile position α to a carbonyl function may lose radioactivity by exchange with protons of the medium during the incubation. For this, an inactivation experiment similar to that in Fig. 4 was set up and samples of the incubation mixture were removed at various times, at once reduced with $NaBH_4$ and freeze-dried. Radioactivity was measured in the residue as well as the volatile fraction. It was found that the specific radioactivity in these two fractions did not change by more than 2% during the entire 3h period needed for 95% inactivation. The possibility was also considered that the radioactivity may be lost from the enzyme–inhibitor complex by an exchange mechanism during the precipitation of the protein and subsequent work-up procedure. For this, in another incubation experiment involving glutamate dehydrogenase and the tritiated inhibitor, samples were removed at 0, 20, 60, 120 and 180min and the radioactivity was stabilized by reducing the glutamate dehydrogenase–inhibitor complex with $NaBH_4$. The complex was then precipitated and processed for the determination of

radioactivity and measurement of protein concentration. The number of mol of the inhibitor incorporated into the protein was the same as that determined without reducing the glutamate dehydrogenase–inhibitor complex. In addition, the pattern and stoicheiometry of the incorporation of the inhibitor into glutamate dehydrogenase was confirmed by using three independently prepared samples of tritiated glutamyl α-chloromethyl ketone and three batches of the enzyme. Despite this care it is a possibility, though a remote one, that some unforeseen artifacts may have contributed to the lower incorporation. The specific radioactivity of the inhibitor was also determined by an independent method described under 'Amino acid analysis of the radioactive peptide'. The information will be used to suggest that the stoicheiometry of the incorporation of radioactivity into the enzyme as determined in Fig. 4 may not be an underestimation by more than 27%.

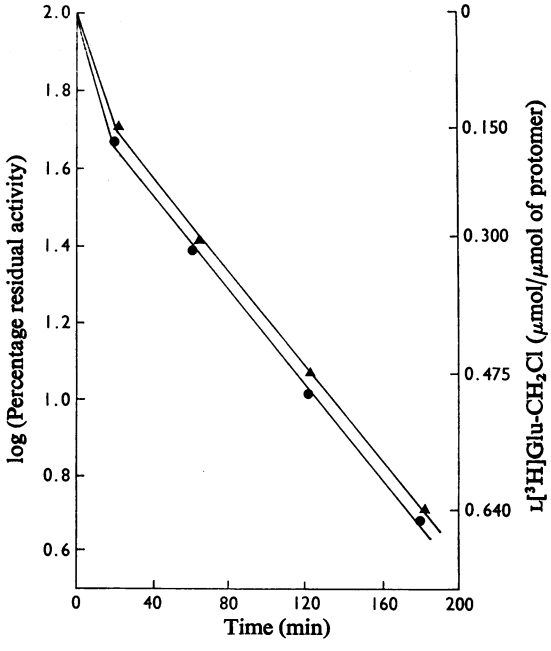


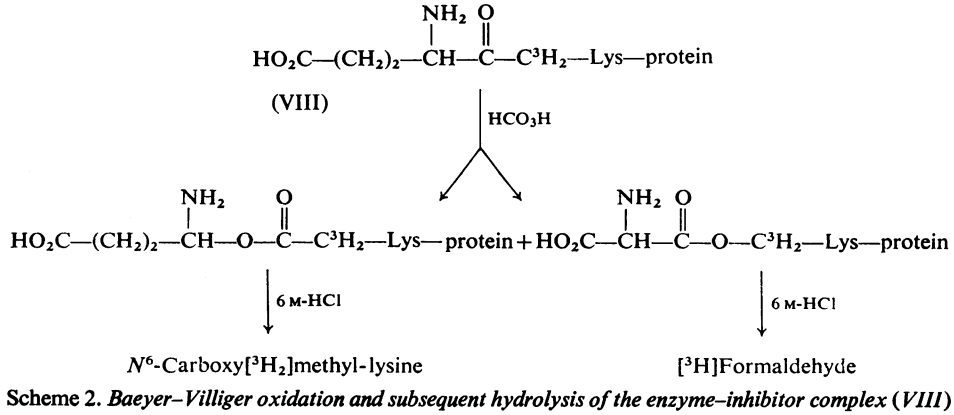
Fig. 4. *Time-course of the inactivation of glutamate dehydrogenase and incorporation of radioactivity from* L-[^3H]*Glu-*CH_2Cl *into the protein*

The enzyme (1.25mg/ml) was incubated with 6mM-L-[^{3}H]Glu-CH_2Cl at 30°C in 0.05M-sodium phosphate buffer, pH6.2. Samples were removed at the times indicated, one (50μl) for enzyme assay and one (0.3ml) to determine the number of μmol of inhibitor incorporated. ●, log (Percentage residual activity); ▲, μmol of the inhibitor incorporated/μmol of the protomer (one molecule of the enzyme consists of six protomers).

Identification of the amino acid residues of glutamate dehydrogenase modified by L-[^3H]*Glu-*CH_2

A 92%-inactivated enzyme obtained as described in the Experimental section was treated with performic acid and then subjected to acid hydrolysis. If we assume that during the course of Baeyer–Villiger oxidation the ^{3}H is not labilized by an enolization mechanism and the insertion of oxygen occurs equally on the two sides of the carbonyl group, then the carboxymethyl amino acid formed through the sequence of Scheme 2 should contain a maximum of 50% of the radioactivity present in the original protein. Under our experimental conditions the residue after oxidation and hydrolysis followed by the removal of volatile radioactivity contained about 21% of the initial radioactivity (i.e. 42% of theory).

A sample of the residue from acid hydrolysis above was analysed by high-voltage paper electrophoresis and more than 70% of the applied radioactivity was present in the region corresponding with the position of N^6-carboxymethyl-lysine. Since under the conditions of electrophoresis N^1-carboxymethylhistidine and N^3-carboxymethylhistidine are poorly separated from N^6-carboxymethyl-lysine, this experiment there-



Scheme 2. *Baeyer–Villiger oxidation and subsequent hydrolysis of the enzyme–inhibitor complex (VIII)*

fore indicates, but does not prove, that the amino acid modified by L-Glu-CH_2Cl is lysine.

Isolation and purification of the 'active site' peptide

The enzyme (40mg) was incubated with 6mM-L-[^{3}H]Glu-CH_2Cl (specific radioactivity 1.4×10^6 c.p.m./μmol) in phosphate buffer, pH6.2. The 96%-inactivated enzyme was reduced with $NaBH_4$ to stabilize the radioactivity and it contained 11135 c.p.m./690μg of protein. It was digested with trypsin and a portion of the tryptic peptides was filtered through a Sephadex G-25 (fine grade) column (see the Experimental section). The elution profile showed the presence of one major radioactive peak containing 65% of the radioactivity applied to the column. The contents of the tubes containing the radioactive peak were freeze-dried. A portion of the labelled peptide (4.5×10^5c.p.m.) was then subjected to high-voltage paper electrophoresis at pH1.9, which showed the presence of many ninhydrin-positive spots, but only one radioactive band; the radioactive band corresponds to a single ninhydrin-positive spot on the guide strip and contained 64% of the radioactivity (2.9×10^5c.p.m.) originally applied to the paper. The radioactive band was found to be homogeneous by further electrophoresis in a buffer of pH5.9. Starting from the modified $NaBH_4$-reduced enzyme, the purified peptide was obtained as a routine in about 30% overall yield.

Amino acid analysis of the radioactive peptide

The amino acid analysis of the radioactive peptide showed that it contained eight amino acid residues in the molar ratios shown in Table 1. Lysine emerged as a relatively broad peak and the integrated value was two amino acid residues. All the radioactivity eluted from the column was also found in this peak suggesting that the modified amino acid co-chromatographed with lysine. This is not surprising, since lysine and the labelled derivative [Scheme 1; (VI)] both contain an excess of one amino group over the carboxyl group(s)

Table 1. *Amino acid composition of the labelled peptide obtained from glutamate dehydrogenase inactivated with* L-[^{3}*H*]*Glu-*CH_2Cl

The amino acid composition was determined as described in the Experimental section. The molar ratios of the amino acids are relative to 1mol of the peptide. The amount of the peptide used for hydrolysis was determined from its specific radioactivity. The hydrolysis was carried out in the presence of a known amount of norleucine. Lys* refers to the modified lysine residue.

Amino acid	Experimental molar ratio	Nearest whole number to experimental
Val	1.01	1
Ala	1.90	1
Gly	3.20	3
Lys + Lys*	2.10	2

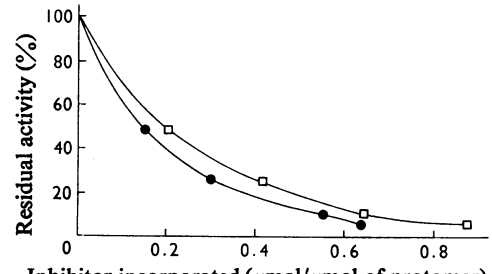


Fig. 5. *Relationship between percentage of the original glutamate dehydrogenase activity remaining and the number of μmol of* L-[^{3}*H*]*Glu-*CH_2Cl *incorporated per μmol of the protomer*

●, Data taken from an experiment of the type described in Fig. 4; □, the above data recalculated assuming that the method underestimated the stoicheiometry by 27%. For a detailed discussion see under the last part of 'Amino acid analysis of the radioactive peptide'.

Table 2. *Sequence of the first three amino acids of the labelled peptide as determined by Edman-subtractive and dansylation methods*

The sequence of the first three amino acids was determined by the Edman-subtraction degradation (Konigsberg, 1967) by using 60nmol of the labelled peptide. The parent peptide and the peptides obtained from the first and second Edman degradations were also subjected to the determination of the *N*-terminal amino acids by the dansylation method (Hartley, 1970). The ratios of the amino acids are relative to 1mol of the appropriate peptide. The amounts of the peptides were determined from their specific radioactivities. Lys* refers to the modified lysine residue.

Edman degradation step	Molar proportions Val:Ala:Gly:(Lys+Lys*)	Amino acid by subtraction	*N*-Terminal amino acid as dansyl derivative
0	1.01:1.90:3.2:2.10	—	Gly
1	1.01:1.70:2.02:2.20	Gly	Gly
2	1.2:1.95:1.15:2.15	Gly	Ala

and therefore should have similar ion-exchange properties. From the knowledge of the specific radio-activity of [^{3}H]Glu-CH_2Cl originally used to modify the enzyme, the total radioactivity eluted from the column corresponded to a molar ratio of 0.73 residue of the modified amino acid/mol of the peptide. This is in satisfactory agreement with the theoretically expected value of 1.0, especially when one considers the number and complexity of steps involved between the original labelling and the final comparison. If, for the sake of argument, we assume that the discrepancy genuinely reflects the experimental error inherent in the approach, then the data of Fig. 4 will be an underestimate by a maximum of 27%. The uppermost limit for the mol of inhibitor incorporated/mol of the enzyme at 20, 60, 120 and 180min will then be 1.2, 2.4, 3.8 and 5.2 respectively. The experimental data of Fig. 4 and the corrected data emerging from the latter consideration are plotted in Fig. 5 to show the relationship between the stoicheiometry of the incorporation of the inhibitor into the protein and the percentage residual activity.

Amino acid sequence of the peptide

The three amino acids of the peptide from the *N*-terminal end were found to be glycine, glycine and alanine respectively (Table 2). This information, when taken in conjunction with its composition, suggested that the peptide corresponded to residues 123–130 [Scheme 1; (VII)] of the sequence of glutamate dehydrogenase (Moon *et al.*, 1972). Since the peptide was the result of tryptic cleavage it is reasonable to assume that its *C*-terminal is the unmodified lysine residue. We may therefore infer that lysine-126 was the modified residue. Lysine-126 has previously been shown to be modified by electrophilic reagents such as pyridoxal phosphate (Piszkiewicz *et al.*, 1970) and 4-iodoacetamidosalicylate at pH7.2 (Holbrook *et al.*, 1973), though the situation with the latter reagent is somewhat more complex. In the earlier work of Malcolm & Radda (1970) inactivation of glutamate dehydrogenase with 4-iodoacetamidosalicylate at pH7.3 was suggested to be due to the alkylation of a cysteine. These workers also reported that in the amino acid analysis of 90%-inactivated enzyme the methionine content was decreased by two residues. The modification of methionine in the inactivation of the enzyme by 4-iodoacetamidosalicylate at pH6.0 has been claimed by Rosen *et al.* (1973).

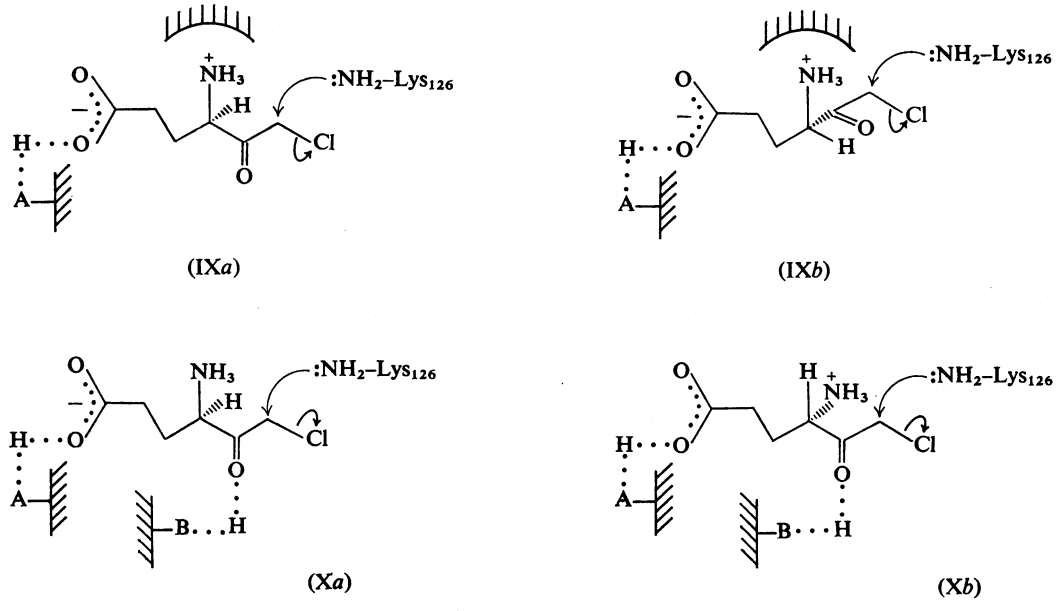
Discussion

It was found that, whereas L-Glu-α-CH_2Cl irreversibly inactivated bovine liver glutamate dehydrogenase, several other chloromethyl ketones, such as L-Glu(γ-CH_2Cl) (V), Ala-CH_2Cl and chloropyruvate, had very little effect on the activity of the enzyme. Substrate specificity for the inactivation suggested that the residue on the enzyme that is modified is near the binding site of the α-carboxyl group of glutamate, because L-Glu(γ-CH_2Cl) (V), which contains the alkylating group towards the other end of the glutamate skeleton, did not inactivate the enzyme. The free γ-carboxyl group of L-Glu-CH_2Cl was also essential for the inactivation of the enzyme, because Ala-CH_2Cl, which lacks the γ-carboxyl group, was without effect.

The lack of stereospecificity with respect to the configuration around the α-carbon atom of the chloromethyl ketones (IV*a*) and (IV*b*) requires comment. During the course of our extensive screening programme on the effect of a large number of glutamate analogues on the activities of six enzymes, which utilize this amino acid as the natural substrate, we have observed a strong preference of the enzyme for a given structure (Rasool, 1975; M. Akhtar, C. G. Rasool & S. Nicolaidis, unpublished work). However, in most cases the inhibitor was non-stereospecific with respect to the carbon atom corresponding to C-2 of glutamate. It therefore appears that when multifunctional compounds such as glutamate are substrates in enzymic reactions, the interaction of all the available groups of the substrate (three in glutamate) with the complementary binding sites on the enzyme is required to produce a fully competent catalytic complex; however, interactions involving fewer sites may result in the formation of enzyme–inhibitor complexes or catalytic complexes with a considerably decreased activity. The fact that L-alanine, having only two of the three important groups of glutamate, is a weak substrate for glutamate dehydrogenase is an example of this phenomenon. In addition, it has been shown that D-glutamate is a potent inhibitor of bovine liver glutamate dehydrogenase (Franco & Iwatsubo, 1972).

The inactivation of glutamate dehydrogenase by the D as well as L isomers of Glu-CH_2Cl may occur by one of two binding modes. First, the interactions involve the γ-carboxyl and the amino groups and the inactivation process depends on the availability of a nucleophilic group accessible to the C–Cl bond of the inhibitors in both the conformations (IX*a*) and (IX*b*) as shown in Scheme 3. Alternatively, the binding results from the interactions of the γ-carboxyl and the oxocarbonyl groups with suitable electrophilic groups on the enzyme to give rise to the complexes (X*a*) and (X*b*). The nucleophilic amino group of lysine-126 is now identically located with respect to the C–Cl bond in both the isomers.

The inactivation of glutamate dehydrogenase by L-Glu-CH_2Cl was pH-dependent. The rate of inactivation increased with decrease in pH (Fig. 6). Since it has been shown that a lysine residue is modified by L-Glu-CH_2Cl, the pH-inactivation profile is in contradiction with the expected behaviour for a nucleophilic



Scheme 3. *Two binding modes of the* D- *and* L-*chloromethyl ketones to glutamate dehydrogenase*

In structures (IX*a*) and (IX*b*) the D and L isomers respectively interact via the γ-carboxyl and the amino groups, whereas in structures (X*a*) and (X*b*) the interactions involve the γ-carboxyl and the oxo groups.

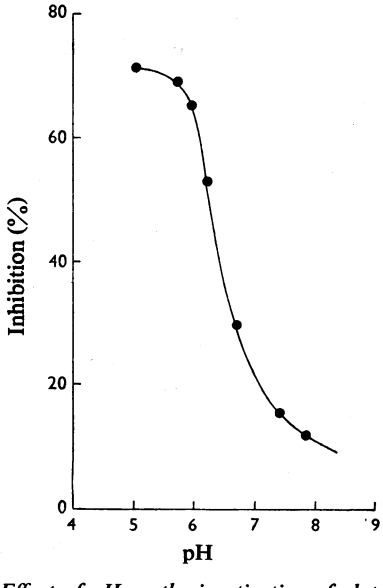
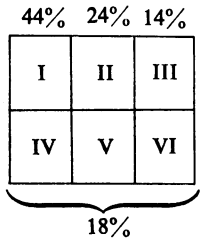


Fig. 6. *Effect of pH on the inactivation of glutamate dehydrogenase by* L-*Glu-CH_2Cl*

The enzyme (0.25mg/ml) was incubated in 0.05M-sodium phosphate buffer (0.5ml) at the pH values indicated. A sample (50μl) was removed after 10 and 20min and assayed by the method described in Fig. 2.

attack by an amino group, which should be favourable only at alkaline pH values. The result, however, may be rationalized by assuming that some electrophilic group(s) on the protein are essential for making hydrogen bonds with the carboxyl and/or the carbonyl groups of L-Glu-CH_2Cl before the nucleophile can attack the chloromethyl ketone moiety, and that the former process is the rate-limiting step in the chemical modification. That the inactivation was optimal at pH values below 7.0 was fortunate and provided some safeguard against the non-specific reactions of protein amino groups with the inhibitor in the labelling experiments described in Fig. 4.

Of all the inhibitors used to date to explore the chemistry of glutamate dehydrogenase it is L-Glu-CH_2Cl that most closely resembles the structure of the substrate. It is therefore tempting to assume that in the enzyme–inhibitor complexes the carbon skeleton of the inhibitor produces some of the subtle interactions normally present in enzyme–glutamate complexes. The study of the relationship between the loss of enzymic activity and the incorporation of radioactivity from [^{3}H]Glu-CH_2Cl into glutamate dehydrogenase showed that the modification of 1.0–1.2 protomers occurred in about 20min and the resulting enzyme, which still contained about five unmodified subunits, had only 50% of the original activity (deduced from Fig. 5 and the time-course data of



Scheme 4. *A hypothetical model showing an asymmetric distribution of enzymic activity between the six subunits of glutamate dehydrogenase*

Subunits I–III containing 82%, and subunits IV–VI only 18% of the total activity [calculated from the data of Fig. 7 (□)]

Fig. 4). In the next 40 min another 1.0–1.2 protomers were modified, and this phase was attended by the additional loss of only 26% of the activity. Further modification had progressively diminishing effects on the loss of the enzymic activity. The results suggest the participation of 'negative co-operative' interactions in the reactivity of lysine-126. Whether the active-site asymmetry giving rise to the 'negative co-operativity' exists in the catalytic hexamer, or results entirely from the chemical modification of subunit(s), is not known at the present time. If it is assumed that the subunit interactions that exist in the enzyme–inhibitor complex are similar to those present in the catalytic Michaelis complex, then, from the cumulative evidence presented above, it can be deduced that the first subunit of the enzyme that takes part in catalysis makes the largest, and the last the smallest, contribution to the overall mechanism. This concept, diagrammatically illustrated in Scheme 4, shows an asymmetric distribution of activity among the subunits of the enzyme and particularly emphasises that one set of three subunits contains a minimum of 82% of the total activity of the enzyme and the remaining set a maximum of only 18%.

In formulating these ideas we are undoubtedly encouraged and influenced by some previous observations. The cross-linking of polypeptide chains of glutamate dehydrogenase by adipimide led Hucho & Janda (1974) to suggest that the hexamer is composed of two sets of three polypeptides. Bell & Dalziel (1972, and references cited therein), on the basis of studies relating to NADH binding in an abortive ternary complex with glutamate, hypothesized that the hexamer may consist of two functionally distinct trimers, which differ in intrinsic affinity for the coenzyme and between them there are negative interactions. Our results point in the same general direction, but further indicate that negative interactions operate not only between, but also within, the trimers. It is interesting to point out that negative co-operativity in the modification of lysine-425 and lysine-428 by trinitrobenzenesulphonate has been observed previously. It was found that the lysine residues in only three of the six subunits of glutamate dehydrogenase were modified by the reagent (Coffee *et al.*, 1971; Goldin & Frieden, 1971). These lysines appear to be associated with the regulatory properties of the enzyme. Further, it was shown that 5′-fluorosulphonylbenzoyladenosine reacted with the second inhibitory NADPH site and that the incorporation of 0.5 mol of the inhibitor per peptide chain abolished the NADPH inhibition (Pranab *et al.*, 1975).

The question may be asked why a similar 'biphasic' inactivation process has not been observed with several other extensively studied reagents that are known to react with the same lysine residue (Lysine-126), which is modified by L-Glu-CH_2Cl. Although an answer based on sound experimentation must await further work, however, it is possible that reagents that give linear inactivation kinetics produce a heterogeneous population of molecules consisting of non-, mono-, di-, tri- etc. alkylated species at all times and the residual enzyme activity measurements give a statistical average of all the molecular species. The phased reactivity observed with Glu-CH_2Cl may be due to the fact that the inhibitor contains a relatively weakly dissociable C–Cl bond, therefore the displacement reaction relies heavily on the activation provided by the interaction of the inhibitor with the active sites of the enzyme, thus revealing their idiosyncracies.

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