The Effect of Modifying Lysine-126 on the Physical, Catalytic and Regulatory Properties of Bovine Liver Glutamate Dehydrogenase

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1. The reaction of 4-iodoacetamidosalicylate with bovine liver glutamate dehydrogenase is dependent on pH. The pH-activity curve is bell-shaped and can be described by apparent pK values of 7.8 ± 0.2 and 9.1 ± 0.2 . 2. Enzyme in which lysine-126 has been modified by 4-iodoacetamidosalicylate has unaltered sedimentation characteristics except when measured in the presence of GTP and NADH. 3. GTP binding to the inhibited enzyme is unaltered. However, GTP can no longer promote the binding of a second molecule of NADH, since this is already bound to the inhibited enzyme without GTP. 4. The equilibrium binding of ADP, GTP, NAD-sulphite and NADH (when measured at low concentrations) was largely unchanged by modification. 5. The number of binding sites for 2-oxoglutarate to the enzyme-NADH complex were decreased by 60% in an enzyme that has been inhibited by 70%.

The preceding paper (Holbrook *et al.*, 1973) describes the labelling of bovine liver glutamate dehydrogenase [L-glutamate-NAD(P) oxidoreductase (deaminating), EC 1.4.1.3] by 4-iodoacetamidosalicylic acid. The inhibitor is specifically incorporated at lysine-126 of the sequence (Moon *et al.*, 1972), causing loss of the enzyme activity. Similar experiments with glutamate dehydrogenase extracted from pig and chicken liver indicate that the part of the sequence around lysine-126 is highly conserved. There is evidence (Baker *et al.*, 1962; Malcolm & Radda, 1970; Roberts, 1970) that 4-iodoacetamidosalicylic acid is reversibly bound at the active site before irreversibly inhibiting the enzyme. Piskiewicz & Smith (1971*a*) have presented data which indicate that lysine-126 has a low pK value (7.9 ± 0.2). Lysine-126 thus appears to be closely associated with the catalytic properties of the enzyme.

Previous experiments have shown that the residual activity of labelled enzyme can still be affected by ADP but that it is no longer sensitive to GTP. This situation is also found when the residual alanine dehydrogenase activity is measured (Malcolm & Radda, 1970).

Colman & Frieden (1966*b,c*) found that acetylation of glutamate dehydrogenase with acetic anhydride under several different conditions resulted in a striking increase in the dissociation constants of the inhibitory substances Zn^{2+} and GTP and an increase in the affinity for ADP. Although the K_m values for all the substrates remained unchanged, the enzymic activity

was lost and the enzyme dissociated. In this case several lysine residues were acetylated and consequently little could be deduced about the function of any one amino group.

More specific lysine reagents have been found and the effects of these on the properties of the enzyme have been investigated. Churchich & Anderson (1966) showed that enzyme that had been inhibited by pyridoxal 5'-phosphate had the same sedimentation coefficient as the oligomer (12–13S) and a tenfold lower affinity for NADH. Pyridoxal 5'-phosphate has been shown to modify lysine-126 (Piskiewicz *et al.*, 1970).

Goldin & Frieden (1972) have investigated the kinetic properties of this modified enzyme and found that the normal inhibition by excess of NADH is almost completely lost when two to three lysine residues per oligomer have reacted. An increase in the K_i for GTP was also found.

The present paper confirms the work of Piskiewicz & Smith (1971*a*) that lysine-126 reacts with an apparent pK of 7.9. The changes in physical, equilibrium-binding and catalytic properties brought about by modification of lysine-126 are discussed. Two reagents, 4-iodoacetamidosalicylate and *N*-(*N*'-acetyl-sulphamoylphenyl)maleimide, have been used to clarify the function of this lysine residue.

Materials and Methods

NAD⁺, NADH, GTP, ADP and 2-oxoglutaric acid were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The sources of

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glutamate dehydrogenase, 4-iodoacetamidosalicylic acid and general laboratory reagents and the methods of enzyme assay and determination of protein concentration are all described in the preceding paper (Holbrook *et al.*, 1973).

Enzyme activity was occasionally measured by the initial rate of oxidation of 0.25 mM-NADH by 0.1 mM-sodium 2-oxoglutarate and 50 mM-NH₄Cl. The reaction was followed at 340 nm with a Hilger-Gilford recording spectrophotometer.

Enzyme was inhibited by incubation of approx. 15 mg of enzyme/ml with 0.8 mM-4-iodoacetamidosalicylic acid in 0.067 M-NaH₂PO₄ buffer adjusted to pH 7.5 with 5 M-NaOH until the required degree of inactivation was obtained. Samples of the incubation mixture were assayed as described in the preceding paper (Holbrook *et al.*, 1973). This method of inhibition resulted in specific alkylation of lysine-126.

The pH-dependence of the rate of reaction of 4-iodoacetamidosalicylate was measured by incubation (20°C) of 0.4 mM-inhibitor with 0.74 mg of enzyme/ml in Na₂H₂P₂O₇ buffers (*I* = 0.75 M) at the appropriate pH. Samples were taken at known time-intervals for assay in the normal way. The pH of the incubation medium was measured before and after the experiment by a Radiometer type 29 pH-meter. The apparent first-order rate constants were obtained from the slopes of plots of the log(residual activity) against time. Enzyme incubated without inhibitor as a control at each pH value was always more than 75% active at the end of the experiments.

The binding of 2-oxoglutarate to the enzyme-NADH complex was studied by the stopped-flow method described by di Franco (1971). When NADH is bound to glutamate dehydrogenase, nucleotide fluorescence is greatly enhanced. However, when 2-oxoglutarate is bound to the highly fluorescent enzyme-NADH complex, the nucleotide fluorescence is quenched. The binding of 4.2 mM-2-oxoglutarate was followed by observing this fluorescence quench when it was rapidly mixed with an equal volume of enzyme (9 μM-subunits of relative molecular mass 56100) containing 92 μM-NADH in a stopped-flow fluorimeter (constructed by Reynolds, 1971). The contents of two syringes containing the reagents were mixed by passing through eight jets into a 1 cm-light-path observation cell. Monochromatic light at 366 nm was passed through the mixed reagents, which were approx. 3 ms old when flow was stopped. The emitted light (at 90° to the incident light) was passed through Wratten 2B and 45 filters and a Corning blue glass filter to remove stray light and was measured by a photomultiplier. The anode current was amplified and displayed on a Tektronix storage oscilloscope. The ambient temperature was about 20°C. The size of the initial rapid transient fluorescence quench is a measure of the concentration of binding sites for 2-oxoglutarate.

The binding of NADH in various complexes was studied initially by using a differential fluorimetric titrator as described by Holbrook (1972) and Holbrook *et al.* (1972). This made use of the changes in NADH fluorescence when excited at 325 nm as NADH is bound to apoenzyme or as other ligands are bound to the enzyme-NADH complex. Results were usually confirmed by the Hummel & Dreyer (1962) method described below.

The binding of the NAD-sulphite complex was studied spectrophotometrically by a method analogous to that used for lactate dehydrogenase (Holbrook & Stinson, 1970). This relied on the absorption of the enzyme-bound complex at 325 nm. Na₂SO₃ (20 mM) was titrated at 5 μl/min into native enzyme (0.88 mg/ml) or enzyme (0.73 mg/ml) that was 62% inhibited by 4-iodoacetamidosalicylate in 10 mM-Tris-2.5 mM-NaH₂PO₄-10 μM-EDTA buffer adjusted to pH 7.2 with 11 M-HCl containing 0.5 mM-NAD⁺. The absorption increase at 325 nm was recorded on a chart recorder and the results were calculated from the maximum absorbance obtained. An extinction coefficient of 5000 litre·mol⁻¹·cm⁻¹ was used (Meyerhof *et al.*, 1938; Gerlach *et al.*, 1966; Holbrook & Stinson, 1970).

The binding of GTP, ADP and NADH was also studied by gel filtration on a column (0.9 cm × 25 cm) of fine-grade Sephadex G-50 by the method of Hummel & Dreyer (1962). Absorption of the eluate was continuously monitored on a Unicam SP.500 modified as a variable-wavelength split-beam continuous-flow spectrophotometer with a linear extinction output to a chart recorder. The amount of ligand bound to the known amount of enzyme on the column was calculated by cutting out the paper corresponding to the trough in the equilibrium concentration of ligand on the column and weighing it. The weight was compared with standard troughs which were obtained by leaving out known quantities of nucleotide from the column buffer.

Sedimentation coefficients were obtained with a Beckman model E analytical ultracentrifuge equipped with schlieren optics. Results were analysed by the method of Schachman (1959) and were corrected for solvent density, viscosity and temperature (20°C) (Svedberg & Pederson, 1940).

The binding of 2-oxoglutarate to glutamate dehydrogenase was also measured by the Hummel & Dreyer (1962) method by using oxo[¹⁴C]glutarate. A stock solution of 15 mM-sodium oxoglutarate in 0.067 M-NaH₂PO₄ buffer adjusted to pH 7.2 with 5 M-NaOH (10 ml) was used to dissolve the contents of a vial containing 20 μCi of 2-oxo[5-¹⁴C]glutaric acid (The Radiochemical Centre, Amersham, Bucks., U.K.). A sample of this solution was diluted exactly tenfold with 67 mM-NaH₂PO₄ buffer, pH 7.2, and was used to equilibrate a column (0.4 cm² × 30 cm) of Sephadex G-25 (fine grade). Samples of enzyme, pre-

pared by mixing 1.35 ml of enzyme (14.5 mg/ml) and exactly 0.15 ml of 15 mM-oxo[^{14}C]glutarate stock solution, were applied to the column, which was then again eluted with the 1.5 mM-oxo[^{14}C]glutarate. Radioactivity in the eluate was monitored with an NE801 (Nuclear Enterprises, Sighthill, Edinburgh, U.K.) detector. ^{14}C radioactivity was counted in an effective volume of about 0.5 ml, with a time-constant of 100 s, an efficiency of 7.5% and (signal+noise)/noise ratio of 2 at 0.4 pCi/ml. The output from the rate-meter was displayed on a chart recorder.

Results

pH-dependence of the 4-iodoacetamidosalicylate reaction

The pH-dependence of the reaction of 4-iodoacetamidosalicylate with glutamate dehydrogenase gives a roughly bell-shaped curve (Fig. 1) with apparent $\text{p}K_a$ values at 7.8 ± 0.2 and 9.1 ± 0.2 . This pH-dependence is not due to changed ionization of the inhibitor molecule, since 4-iodoacetamidosalicylate has no groups that titrate in the pH range used. By using 4-iodo[^{14}C]acetamidosalicylate it was found that the reaction was still as specific for lysine at both pH 8.5 and 9.0 as it was at pH 7.5. Carboxymethyl-lysine was isolated after acid hydrolysis of the protein by using the method described in the preceding paper (Holbrook *et al.*, 1973). Since 30 mM-2-oxoglutarate prevented inhibition at pH 8.5 as it did at pH 7.5, it is probable that the lysine alkylated is lysine-126 at all the pH values used. Both the apparent $\text{p}K_a$ values found are therefore ascribed to ionization of the enzyme molecule.

The variation with pH of the equilibrium constant for the formation of a reversible enzyme-inhibitor complex is also shown in Fig. 1. 4-Iodoacetamidosalicylate does not reversibly bind to the enzyme at pH 8.5, the pH at which the covalent reaction of enzyme with inhibitor proceeds most rapidly. Thus, the pH-dependence of the rate of inhibition by 4-iodoacetamidosalicylate is not caused by a difference in ionization state of the residues that reversibly bind the inhibitor. The $\text{p}K$ of 7.8 could therefore reflect ionization of a group or system involving lysine-126 itself, but it would be difficult to exclude the possibility that this was the $\text{p}K$ of a system controlling access to the lysine residue. The decrease in reactivity of lysine-126 at high pH values ($\text{p}K = 9.1 \pm 0.2$) is not dissimilar to the decrease in enzymic activity ($\text{p}K = 9.5 \pm 0.2$) as the pH is raised. The pH-dependence of the inhibition reaction does not coincide with the pH-dependence of the enzyme activity when assayed in either direction, apart from that discussed above (Wallis & Holbrook, 1972).

Fig. 1. Comparison of the pH-dependence of 4-iodoacetamidosalicylate inhibition with that of the pre-binding of the inhibitor

The pH-dependence of the reaction of 4-iodoacetamidosalicylate with glutamate dehydrogenase ($\circ$) was obtained by assaying 5 μl samples of an incubation mixture containing 0.405 mM-inhibitor and 0.74 mg of enzyme/ml. The buffers were sodium pyrophosphate of constant ionic strength ($I = 0.75 \text{ M}$). Incubation was at 25°C . The results are expressed as the first-order rate constant of inactivation (k). The K_i for prebinding of inhibitor to enzyme was obtained from the competitive inhibition of 4-iodoacetamidosalicylate and 2-oxoglutarate (Δ). Enzyme activity was assayed in cuvettes containing 2-oxoglutarate (0.05–0.2 mM), 4-iodoacetamidosalicylate (0–9.0 mM), 0.25 mM-NADH and 50 mM- NH_4Cl . The same pyrophosphate buffers were used. Results were analysed by the method of Dixon & Webb (1958).

Effect of labelling on polymerization of the enzyme

Inhibition by 4-iodoacetamidosalicylate has little effect on the state of association of the enzyme under a variety of conditions (Table 1). However, enzyme that was inhibited by 70% exhibited two distinct sedimenting components when measured in the presence of GTP and NADH. One of these corresponded to the oligomer (13.4S). The higher-molecular-weight form was probably not saturated with GTP, since Colman & Frieden (1966a) have observed a similar phenomenon when low concentrations of GTP (compared with the dissociation constant) were used.

Effect of labelling on the binding of 2-oxoglutarate

2-Oxoglutarate competes with 4-iodoacetamidosalicylate (Baker *et al.*, 1962). A more-detailed examination of the interrelations of 2-oxoglutarate binding and lysine-126 was carried out by using three reagents that label this group. Malcolm & Radda (1970) have shown that 2-oxoglutarate prevents inhibition when enzyme is incubated with 4-iodoacetamidosalicylate. Our work confirms this (Fig. 2). The rate of reaction of *N*-(*N*'-acetylsulphamoylphenyl)maleimide with lysine-126 is also decreased in the presence of 2-oxoglutarate. The first-order rate constant of reaction of 2mM-inhibitor with glutamate dehydrogenase (2.2mg/ml) at 25°C in 0.1M-NaH₂PO₄ buffer adjusted to pH7.5 with 5M-NaOH is decreased from 0.017min⁻¹ to 0.002min⁻¹ by inclusion of 10mM-2-oxoglutarate in the incubation (J. J. Holbrook, unpublished work). It therefore seemed likely that lysine-126 could be prevented from reaction with pyridoxal 5'-phosphate by 2-oxoglutarate. By using a very low concentration of this substrate (5mM) Piskiewicz & Smith (1971b) were unable to demonstrate protection. Fig. 3 shows a similar experiment where the pyridoxal 5'-phosphate concentration is lower and the 2-oxoglutarate concentration higher (30mM). This concentration of substrate would be expected to 90% saturate the binding site ($K_s=4.3$ mM; Malcolm & Radda, 1970) and consequently decrease the rate of inhibition of the enzyme by 90% if 2-oxoglutarate and pyridoxal 5'-phosphate bind at the same site. 2-Oxoglutarate clearly protects the enzyme against inhibition and these results are therefore in disagreement with the work of Piskiewicz & Smith (1971b). These workers, however, would not have found much protection since the concentration of substrate was too low and that of inhibitor too high.

The behaviour of 2mM-4-iodoacetamidosalicylate during electrophoresis at pH6.5 was not changed by incubation with 30mM-2-oxoglutarate for 30min at pH7.5 (in 0.067M-NaH₂PO₄ buffer adjusted to

pH7.5 with 5M-NaOH). The protection caused by 2-oxoglutarate is therefore due to this substrate binding to the inhibitor.

To explain these results it is necessary that 2-oxoglutarate can bind to the apoenzyme. The Hummel & Dreyer (1962) method was once again employed. A

Fig. 2. Protection by 2-oxoglutarate of glutamate dehydrogenase from inhibition by 4-iodoacetamidosalicylate

Enzyme (3mg/ml) was incubated (37°C) either alone (■), with 2mM-4-iodoacetamidosalicylate (▲) or with 2mM-4-iodoacetamidosalicylate and 34mM-2-oxoglutarate (△). Samples (5μl) were taken at the times indicated and assayed for enzyme activity. The buffer was 0.067M-NaH₂PO₄ adjusted to pH7.5 with 5M-NaOH.

Table 1. Sedimentation coefficients of native and iodoacetamidosalicylate-inhibited glutamate dehydrogenases

Values were obtained by centrifuging glutamate dehydrogenase (1.58mg/ml) either alone or with the nucleotides indicated. The normal buffer was 0.1M-NaH₂PO₄ adjusted to pH7.0 with 5M-NaOH. The high-salt buffer was 0.2M-NaH₂PO₄ containing 0.1M-NaCl adjusted to pH7.0. The rotor speed was 52640rev./min at 20°C. Values are corrected for solvent density and viscosity.

Conditions	Sedimentation coefficients (S)		
	Native enzyme	30%-iodoacetamidosalicylate-inhibited enzyme	70%-iodoacetamidosalicylate-inhibited enzyme
Alone	24.2	23.2	23.0
In 1mM-GTP + 1mM-NADH	14.1	14.1	13.4 and 19.1
In 1mM-ADP + 1mM-NADH	21.7	21.6	20.8
In high-salt buffer	21.8	Not determined	22.1

Fig. 3. Protection by 2-oxoglutarate of glutamate dehydrogenase from inhibition by pyridoxal 5'-phosphate

Enzyme (1.85 mg/ml) was incubated (20°C) either alone (■), with 80 μM-pyridoxal 5'-phosphate (▲), or with 80 μM-pyridoxal 5'-phosphate plus 30 mM-2-oxoglutarate (△). The method used was as described in Fig. 2. The buffer was 0.067 M-NaH₂PO₄ adjusted to pH 7.2 with 5 M-NaOH.

buffer of 1.5 mM-sodium 2-oxo[5-¹⁴C]glutarate was used to equilibrate the column. The enzyme was eluted with a peak of radioactivity about 3% above the equilibrium value and was followed by a trough about 3% below the equilibrium counting rate. From the chart speed, flow rate of the column (14 ml · h⁻¹) and the equilibrium concentration of 2-oxoglutarate on the column, the amount of enzyme-bound oxo[¹⁴C]glutarate was calculated from both the area of the peak and the area of the trough. In two experiments 378 nmol of enzyme polypeptide bound 122 nmol of 2-oxoglutarate (from troughs) and 107 nmol (from peaks). As the peaks are subject to less errors than the troughs, the enzyme subunits were about 28% saturated. This is consistent with a minimum oxoglutarate dissociation constant of 4 mM in the absence of any other ligand.

An investigation of the effect of covalently bound 4-iodoacetamidosalicylate on the binding of 2-oxoglutarate was also carried out. Fig. 4 shows that the rapid quench of NADH fluorescence as 2-oxoglutarate binds to the enzyme-NADH complex is significantly changed by the presence of the inhibitor. In fact, 30%-active enzyme only displays 40% of the change observed with fully active enzyme. This lower value is not apparently due to a change in the affinity of the enzyme for 2-oxoglutarate, since the same

Fig. 4. Effect of 4-iodoacetamidosalicylate inhibition on the binding of 2-oxoglutarate to the enzyme-NADH complex

2-Oxoglutarate (4.18 mM) was mixed with an equal volume of 92 μM-NADH containing enzyme in a stopped-flow fluorimeter. The rapid quench in NADH fluorescence is a measure of the amount of oxoglutarate bound to the enzyme. The trace in (a) was produced by using a final concentration of native enzyme of 4.85 μM-polypeptide chains and the trace in (b) by a final concentration of 4.2 μM-inhibited enzyme (70% inactivated by iodoacetamidosalicylate). Vertical axis 10 mV/division; horizontal axis 20 ms/division.

result was obtained when the concentration of this substrate was raised from 2.1 mM to 34.2 mM.

Effect of labelling on the binding of nicotinamide nucleotides

When measured by fluorimetric titration, the affinity of the enzyme for NADH was unaltered by inhibition of the enzyme by 4-iodoacetamidosalicylate. The binding constant for NADH in 10 mM-Tris buffer adjusted to pH 7.2 with 11 M-HCl was 2.4 μM with the native enzyme and 2.9 μM with the 67%-inhibited enzyme.

Gel filtration by the Hummel & Dreyer (1962) method by using $60\text{ }\mu\text{M}$ -NADH showed that although the native enzyme could bind 5.7 mol of NADH/mol of oligomer, the 68% -inhibited enzyme could bind 5.4 mol of NADH/mol of oligomer. This is the same within the experimental error. These results have been corrected for any residual NADH bound to the enzyme before the experiment. This was measured by the change in E_{340} when a large quantity (4 mg) of

enzyme was mixed with excess of diammonium 2-oxoglutarate ($50\text{ }\mu\text{M}$) in the spectrophotometer cuvette. The correction amounted to less than 5% of the NADH bound to the enzyme.

A similar experiment carried out with a high concentration of NADH (1 mM) yielded entirely different results (Table 2). The native enzyme only bound one molecule of NADH but there are two NADH-binding sites occupied per subunit of inhibited

Table 2. *Effect of labelling glutamate dehydrogenase with 4-iodoacetamidosalicylate on the binding of GTP and NADH*

Values are expressed as the number of mol of nucleotide bound/mol of glutamate dehydrogenase oligomer. These were obtained by the Hummel & Dreyer (1962) gel-filtration method described in the text. NADH+GTP binding was measured by absorption at 252 nm , and NADH binding alone was measured at 340 nm .

Nucleotide bound	Concn. of ligand	Nucleotide bound	
		Native enzyme	60% -iodoacetamidosalicylate-inhibited enzyme
NADH+GTP	$1\text{ mM-GTP} + 1\text{ mM-NADH}$	18.1	16.9
NADH	$1\text{ mM-GTP} + 1\text{ mM-NADH}$	10.9	13.2
GTP (by subtraction)	$1\text{ mM-GTP} + 1\text{ mM-NADH}$	7.2	3.7
NADH	1 mM-NADH	7.6	15.8

Fig. 5. *Effect of 4-iodoacetamidosalicylate inhibition on the binding of ADP*

Binding results were obtained by gel filtration on a column ($0.9\text{ cm} \times 25\text{ cm}$) of fine-grade Sephadex G-50 (Hummel & Dreyer, 1962). A known quantity of enzyme ($2\text{--}7\text{ mg}$) was applied in 0.8 ml of buffer containing ADP to the column, which had been equilibrated with the same total concentration of ADP. The amount of ADP bound to the enzyme was calculated from the trough in equilibrium concentration of ADP when measured by absorption at 260 nm . Results are presented in a double-reciprocal plot. ∇ , Native enzyme; $\square$, enzyme 65% inhibited with 4-iodoacetamidosalicylate.

enzyme. One has a very high affinity ($K = 2.9 \mu\text{M}$). The other has a lower affinity, since occupation of the second site cannot be detected at $60 \mu\text{M}$ -NADH. Unfortunately, there is a large inherent error ($\pm 20\%$) in these results owing to the large background absorbance of the NADH, even in a 1 mm-path-length cuvette.

Measurement of the number of NAD⁺ binding sites present in native and inhibited enzyme was carried out by applying the NAD-sulphite method (Holbrook & Stinson, 1970). Whereas native enzyme had 5.6 binding sites for the NAD-sulphite complex per oligomer, the 62%-inhibited enzyme had 4.2 binding sites.

Effect of labelling on the binding of nucleotide effectors

The binding of ADP was studied by the Hummel & Dreyer (1962) method in 0.1 M-NaH₂PO₄ buffer adjusted to pH 7.0 with 5 M-NaOH, by using various ADP concentrations. Double-reciprocal plots of the results (Fig. 5) show that very little change in either

Fig. 6. *Effect of 4-iodoacetamidosalicylate inhibition on the GTP-induced enhancement of enzyme-bound NADH fluorescence*

Curve A was produced by titrating GTP at $8.3 \mu\text{M}/\text{min}$ into a cuvette containing $1.2 \mu\text{M}$ -subunits of native enzyme and $13 \mu\text{M}$ -NADH. Curve B was produced by titrating GTP at $83 \mu\text{M}/\text{min}$ into a cuvette containing $13 \mu\text{M}$ -NADH and $5.9 \mu\text{M}$ -subunits of enzyme which was 72% inhibited by 4-iodoacetamidosalicylate. The buffer used was 0.067 M-NaH₂PO₄ adjusted to pH 7.2 with 5 M-NaOH. Titration began at the times indicated. The relative amplification was the same for both experiments.

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the binding constant ($4.2 \mu\text{M}$) or the number of binding sites (6.1 binding sites per oligomer) has been caused by inhibition of the enzyme.

When GTP binds to the glutamate dehydrogenase-NADH complex it co-operatively enhances the already large nucleotide fluorescence. For this reason GTP binding was initially studied by the fluorescence-titration method (Fig. 6). The extent of change in fluorescence was measured and it was found that enzyme that was 72% inhibited had only 13% of the fluorescence change induced in the native enzyme. Thus, the GTP effect on NADH fluorescence is lost, as is the GTP inhibition of the residual enzyme activity.

The binding of GTP to the enzyme was also investigated by the more direct method of Hummel & Dreyer (1962), by using various concentrations of GTP. A double-reciprocal plot of the results is shown in Fig. 7. The number of GTP-binding sites per oligomer remains the same after inhibition (5.6). However, the affinity of the enzyme for GTP is greatly increased (the dissociation constant decreases from 52.6 to $18.9 \mu\text{M}$) by modification of lysine-126. Thus, although the effects of GTP are lost on modification, this nucleotide is still bound very tightly. The reason that 0.1 M-Tris-HCl buffer was used in this experiment was that the GTP dissociation constant when

Fig. 7. *Effect of 4-iodoacetamidosalicylate inhibition on the binding of GTP*

Results were obtained by the Hummel & Dreyer (1962) method as described in Fig. 5 except that GTP replaced ADP. Results are presented as a double-reciprocal plot. ▽, Native enzyme; □, 85%-inhibited enzyme.

measured in 0.1M-sodium phosphate buffer at the same pH (pH 7.2) is approx. 0.7mM for the native enzyme (Wallis, 1972).

The Hummel & Dreyer (1962) method was also used to attempt to measure the binding of GTP and NADH together. In this case the column was equilibrated with 1mM-NADH and 1mM-GTP together. The binding of both nucleotides was monitored at 252nm. In a separate experiment, NADH binding (also in the presence of GTP) was measured at 340nm, a wavelength where GTP has a very small absorbance. Each individual result has a large possible error ($\pm 15\%$) owing to the large background absorbance. Thus the values for GTP bound in the presence of NADH, obtained by subtracting the value for NADH bound (determined from E_{340}) from the value for GTP+NADH bound (from E_{252}), have an even larger possible error. The results are summarized in Table 2.

Discussion

It is reasonable to assume that the reaction of 4-iodoacetamidosalicylate with lysine-126 proceeds as nucleophilic attack of the unprotonated lysine ϵ -amino group on the slightly electropositive methylene carbon of the inhibitor. Thus reaction is favoured by high pH values.

Lysine-126 is very susceptible to attack by alkylating agents at neutral pH values. The possibility that it has an unusually low pK value was considered by Holbrook & Jeckel (1969). Piskiewicz & Smith (1971a) have shown that the rate of reaction of pyridoxal with lysine-126 to form a Schiff base with glutamate dehydrogenase can be described by a pK of 7.9 ± 0.2 . This is the same pK as one of those that describe the reaction of 4-iodoacetamidosalicylate with the enzyme (7.8 ± 0.2). Although it is tempting to conclude that this important lysine residue does have such a low pK, the possibility that the apparent pK represents ionization of a group controlling access of the two reagents to this lysine has yet to be excluded.

Inhibition of the enzymic activity by covalently bound 4-iodoacetamidosalicylate is not due to dissociation of the enzyme into subunits. The binding of effector and substrate molecules was measured to see if these were changed by the lysine modification. The binding and effect of ADP is unaltered by modification. The binding of GTP, NAD^+ and NADH at low concentrations is also changed very little by modification by 4-iodoacetamidosalicylate. There are, however, two major differences between native and inhibited enzyme. The first is that the substrate, 2-oxoglutarate, causes very little quenching of the fluorescence of NADH bound to the inhibited enzyme. The most likely explanation of this is that 2-oxoglutarate does not bind to enzyme subunits

which have an alkylated lysine-126. This in itself would cause loss of enzymic activity.

Preliminary steady-state kinetic data (Engel & Dalziel, 1970) indicate that 2-oxoglutarate can bind to the unliganded enzyme, in contrast with most other dehydrogenases, where NADH must bind before the other substrate (Takenaka & Schwert, 1956; Theorell & Chance, 1951). In the present paper we have given direct evidence that 2-oxoglutarate can bind before NADH. Baker *et al.* (1962) have shown competition between 4-iodoacetamidosalicylate and 2-oxoglutarate. It is likely that the reason for the decrease in 2-oxoglutarate-binding sites in the inhibited enzyme is due to the presence of a blocked lysine-126 at the 2-oxoglutarate-binding site.

The second major change in properties caused by 4-iodoacetamidosalicylate involves the binding and effects of NADH and GTP. Jallon & Iwatsubo (1971) and Koberstein & Sund (1971) have shown, by difference spectroscopy, that there are two binding sites for NADH when GTP is bound to the enzyme. The results in the present paper confirm this by the more direct Hummel & Dreyer (1962) method. When the enzyme is inhibited by 4-iodoacetamidosalicylate the second NADH-binding site can be occupied in the absence of GTP (as well as in its presence). 4-Iodoacetamidosalicylate thus appears to induce the conformation change normally caused by GTP which enables the second NADH to bind with low affinity. However, GTP can still bind normally to the apoenzyme. Another linkage between GTP and NADH binding is changed by modification. This is the ability of bound GTP to cause a change in bound NADH fluorescence. This is virtually abolished by 4-iodoacetamidosalicylate. These changes might be sufficient to explain the inhibition of the enzymic activity, since the inhibition could lock the enzyme into the inactive conformation normally favoured when GTP binds. However the GTP-induced form exists as the oligomer (14.1S) (Colman & Frieden, 1966a) and has an activated alanine dehydrogenase activity (Yielding *et al.*, 1964). The enzyme which is modified by 4-iodoacetamidosalicylate exists in an associated form (23.0S) and the alanine dehydrogenase activity is as inhibited as the glutamate dehydrogenase activity (Malcolm & Radda, 1970). Thus, this is not a likely explanation for the loss of activity.

N-(*N*'-Acetylsulphamoylphenyl)maleimide also labels lysine-126 (Holbrook & Jeckel, 1969) and imparts very similar properties on the enzyme to those produced by 4-iodoacetamidosalicylate (J. J. Holbrook, unpublished work). Table 3 summarizes these results. The most striking feature of these is the dramatic increase in K_i for GTP when measured by steady-state kinetics. It is also noteworthy that high concentrations of NAD^+ impart a similar percentage activation to both native and inhibited enzyme. This

Table 3. *Effect of labelling lysine-126 of glutamate dehydrogenase with N-(N'-acetylsulphamoylphenyl)maleimide on the steady-state properties of the enzyme*

Inhibited enzyme was prepared by incubating enzyme (5 mg/ml) with 0.7 mM of the inhibitor in 67 mM- NaH_2PO_4 buffer adjusted to pH 7.5 with 5 M-NaOH for 6 h. The solution was filtered through Sephadex G-50 in the same buffer to remove unchanged inhibitor (Holbrook & Jeckel, 1969). Control enzyme was treated similarly but without the maleimide. Assays were in 50 mM-Tris buffer adjusted to pH 8.0 with 11 M-HCl and the production of NADH was followed spectrophotometrically at 340 nm. $V_{\max}$ and K_m for glutamate were measured by using 0.5 mM-NAD⁺ and various potassium glutamate concentrations from 0.1 mM to 2.5 mM. The K_m for NAD⁺ was determined by using 8.65 mM-potassium glutamate and various NAD⁺ concentrations from 10 to 50 μM . The activation by high NAD⁺ concentration was the ratio of $V_{\max}$ at high NAD⁺ concentration (100–500 μM). K_i for GTP was determined by using 8.65 mM-glutamate and 1 mM-NAD⁺. The GTP concentration was varied from 0.2 to 2 μM for native enzyme and from 2 to 100 μM for inhibited enzyme. K_i was taken as the concentration of GTP required for half-maximum inhibition. ADP activation was the increased rate caused by ADP (0.5 mM) in the assay medium (8.65 mM-glutamate, 0.05 mM-NAD⁺ in the Tris-HCl buffer, pH 8.0).

	Native enzyme	N-(N'-Acetylsulphamoylphenyl)-maleimide-inhibited enzyme
Relative $V_{\max}$.*	1	0.21
K_m (glutamate)	1.3 mM	1.5 mM
K_m (NAD ⁺)	37 μM	27 μM
Activation by high NAD ⁺	2.4-fold	2.6-fold
K_i (GTP)	0.4 μM	5 μM
Activation by ADP	2.0-fold	1.8-fold

* The absolute activity for native enzyme was 5 μmol of glutamate transformed/min per mg.

indicates that the inhibition by this reagent is not simply due to loss of NAD⁺ activation. This is substantiated by the fact that inactivation by 4-iodo-acetamidosalicylate proceeds at an identical rate if the NAD⁺ in the assay medium is replaced with NADP⁺. NADP⁺ causes no activation of the enzyme (C. Frieden, personal communication).

These results are all consistent with the hypothesis that glutamate dehydrogenase in which lysine-126 is modified is inactive because 2-oxoglutarate cannot bind to the catalytic site. As a secondary consequence of modification the co-operative linkage between GTP and the binding of the second, inhibitory, molecule of NADH to each subunit is lost.

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