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A Peptide Containing a Reactive Lysyl Group from Ox Liver Glutamate Dehydrogenase

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1. Inhibition of ox liver glutamate dehydrogenase with *N*-(*N'*-acetyl-4[^{35}S]-sulphamoylphenyl)maleimide (ASPM) is more specific at pH 7·3 than at pH 6·9. At pH 7·3 inhibition accompanies the incorporation at 1 mole of ASPM residues into about 53000g. of protein. 2. Digestion of the modified protein with chymotrypsin and trypsin yields a unique radioactive peptide. 3. Acid hydrolysis of 1 mole of this peptide yields 1 mole of N^{ϵ}-succin-2-yl-lysine. The ϵ-amino group of a lysyl residue is thus the site of modification of the protein. 4. The sequence containing the modified lysyl residue is:

ASPM
|
(Ala,Asx_2,Val_3)-Phe-Gly-Ala-(Gly,Lys)-Ala-Gly-Val-Lys

where Asx respresents either aspartic acid or asparagine.

The similarities in the amino acid sequences that contain the essential thiol groups of dehydrogenases of differing substrate specificities (Holbrook *et al.* 1967) made it desirable to extend the range of dehydrogenases examined. The usefulness of ASPM* as a reagent for specifically labelling the essential thiol group of lactate dehydrogenase (Holbrook & Pfleiderer, 1965) suggested its application to other dehydrogenases. Of the other dehydrogenases examined in these Laboratories (mitochondrial and cytoplasmic malate dehydrogenases, α-glycerophosphate dehydrogenase and GDH) only with GDH was any specificity in the labelling detected (Pfleiderer, Holbrook, Nowicki & Jeckel, 1966). These preliminary results indicated that the amino acid that reacts with ASPM in GDH at pH 6·9 is cysteine. The complete results presented in this paper show that at pH 7·3 the reaction is more specific and prove that the site of modification is in fact an ϵ-amino group of a lysyl residue and not the thiol group of cysteine.

Treatment of GDH with such reagents as acetic anhydride (Colman & Frieden, 1966*a*,*b*) or pyridoxal phosphate (Anderson, Anderson & Churchich, 1966) results in an inhibited enzyme preparation that has lost its ability to aggregate at high protein concentration and whose residual enzyme activity does not respond to effectors. Although both acetic anhydride and pyridoxal phosphate normally react with the amino groups of proteins neither reagent is so specific that reaction with other side chains may be easily excluded. Equally, without the use of labelled modifying agents, it is difficult to be certain of the number of amino groups that must be modified to achieve the changes in hydrodynamic and ligand-binding properties observed. Measurement of these properties in GDH preparations in which a unique lysine residue has reacted with ASPM should remove some of these uncertainties (J. J. Holbrook & R. C. Cantwell, unpublished work).

Abbreviations: ASPM, *N*-(*N'*-acetyl-4-sulphamoylphenyl)maleimide; GDH, glutamate dehydrogenase (EC 1.4.1.3); 'dansyl', 1-dimethylaminonaphthalene-5-sulphonyl.

MATERIALS AND METHODS

Materials. *N*-(*N'*-Acetyl-4[^{35}S]-sulphamoylphenyl)-maleimide was purchased from Radiochemischeslabor, Farbwerke–Hoechst A.-G., Hoechst, Germany (specific radioactivity 5mc/m-mole). Ox liver GDH, NAD^+, NADH, GTP and ADP were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Carboxypeptidase B was a product of Worthington Biochemical Corp., Freehold, N.J., U.S.A. Trypsin, chymotrypsin, Dowex 1 and Dowex 50W (X2) were bought from Serva Entwicklungslabor, Heidelberg, Germany. Sephadex products were from Pharmacia, Uppsala, Sweden. N^{α}-Toluene-*p*-sulphonyl-lysine and N^{α}-acetylhistidine were obtained from

Cyclo Chemical Corp., Los Angeles, Calif., U.S.A. *N*-Ethylmaleimide was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. The subtilopeptidase preparation, under the name Alcalase, was a gift from Novo Industri A/S, Copenhagen, Denmark.

Methods. Most of the techniques, apparatus and solvents used in the thin-layer chromatography and electrophoresis of amino acids and peptides were as described by Holbrook, Pfleiderer, Schnetger & Diemair (1966). The supporting medium was a 0·325mm.-thick layer of Kieselgel–starch, unless otherwise stated. The radioactivity in the eluates from chromatographic columns was continuously monitored with an NE5504/NE806 flow-through scintillation counter (Nuclear Enterprises Ltd., Edinburgh). Ultraviolet absorption of the eluates was also continuously monitored with a Uvicord I (LKB-Produkter AB, Stockholm, Sweden). GDH protein concentrations were determined by the biuret method, the conversion factor 19·5 being used (Sund & Åkeson, 1964). The amount of ASPM incorporated into GDH was calculated from the radioactivity as determined by using a gas counter as described for lactate dehydrogenase by Holbrook *et al.* (1966). N^{ϵ}-Succin-2-yl-lysine was synthesized from N^{α}-toluene-*p*-sulphonyl-lysine and *N*-ethylmaleimide by the method of Brewer & Riehm (1967). The succinyl-lysine was separated from the ethylamine and lysine present after the hydrolysis with 6N-HCl by electrophoresis at pH6·5. *N*-Succin-2-ylhistidine was prepared and purified in an analogous manner. *S*-Succin-2-ylcysteine was prepared from the acid hydrolysate of the products of the reaction of *N*-ethylmaleimide with cysteine as described by Smyth, Blumenfeld & Konigsberg (1964). The 'dansyl'–Edman method (Gray & Hartley, 1963) was used to degrade peptides from their *N*-termini. In some cases the paper-strip modification (Schroeder, Shelton, Shelton, Cormick & Jones, 1963) of the Edman degradation was employed. The phenylthiohydantoin derivatives of amino acids were identified by thin-layer chromatography (Brenner, Niederwieser & Pataki, 1962). GDH enzyme activity was assayed as the rate of reduction, at 25°, of 1mM-NAD^+ by 17mM-potassium glutamate in 67mM-KH_2PO_4 adjusted to pH7·3 with 5N-NaOH.

Peptides were hydrolysed in constant-boiling HCl in sealed tubes at 110° for 48hr. The amino acids produced were determined by using a Technicon amino acid analyser (Technicon, Chertsey, Surrey). Peptide 'maps' were prepared by using the thin-layer system of Wieland & Georgopoulos (1964). Radioactive material was detected by laying the plates on X-ray photographic paper.

Inhibition of GDH with ASPM. The GDH enzymic activity of a solution (200ml.) of GDH (5·02mg./ml.) and ASPM (1·94mM) incubated at 25° in 67mM-KH_2PO_4 adjusted to pH7·3 with 5N-NaOH was periodically assayed. After 85min., when 67% of the initial activity had been destroyed, the reaction was terminated by the addition of 2-mercaptoethanol (0·1ml.) and the reaction mixture was filtered in two portions (100ml. each) through a column (2·4cm.×100cm.) of Sephadex G-50 (coarse grade) that had been equilibrated with 50mM-NH_4HCO_3. The protein peaks were pooled, and samples were taken to determine the protein concentration and the radioactivity; the ASPM–GDH was recovered from the remainder (250ml.) by the addition of 3M-trichloroacetic acid (10ml.). The precipitate was washed with 10mM-trichloroacetic acid, acetone and then ether, and finally dried. The yield was 870mg.

Hydrolysis of ASPM–GDH with trypsin or with trypsin and chymotrypsin. ASPM–GDH (850mg.) was dispersed in 2mM-HCl (50ml.) by means of a motor-driven Potter–Elvehjem homogenizer. The suspension, pH3·2, was heated on a boiling-water bath for 5min., when most of the protein dissolved. The ASPM–GDH was reprecipitated in a flocculent form by the addition of NH_4HCO_3 (0·7g.). Trypsin (14mg.) alone, or trypsin (14mg.) and chymotrypsin (14mg.), was added to the suspension, which was gently stirred for 3hr. at room temperature. Solution was 95% complete after 1hr. The solution was heated on a boiling-water bath for 10min. The denatured proteolytic enzymes, together with 4% of the total radioactivity, were centrifuged down and discarded. Samples of the supernatant were taken for peptide 'mapping'. The supernatant was concentrated to about 7ml. by rotary evaporation at 40°.

Preparation of a labelled tryptic peptide. A solution of the soluble peptides from a tryptic digestion of ASPM–GDH (600mg.) was filtered through a column (2·6cm.×80cm.) of Sephadex G-25 (fine grade) in 10mM-NH_4HCO_3. The major radioactive peak, containing 87% of the eluted ^{35}S, which was eluted just after the exclusion volume of the column, was further purified by ion-exchange chromatography on Dowex 1 by using the system described below for peptide ASPM–GDH-TC-1. The radioactive material eluted at pH3·9 was further purified by thin-layer chromatography in butan-1-ol–pyridine–acetic acid–water (68:40:14:25, by vol.) and electrophoresis at pH6·5. The radioactive compound obtained migrated with the same speed as the only compound in the preparation that reacted with ninhydrin. The peptide was designated ASPM–GDH-T-1.

Preparation of the labelled tryptic–chymotryptic peptide. The soluble peptides from a combined tryptic–chymotryptic digestion of ASPM–GDH (850mg.) were applied to a column of Sephadex G-25 as described in Fig. 1. Fractions 63–78 were pooled, concentrated by rotary evaporation and freeze-dried. The residue was chromatographed on a column (1cm.×100cm.) of Dowex 1 under the conditions described by Schroeder & Robberson (1965). The radioactive material eluted between pH6·0 and 5·8 (Fig. 2) was recovered by freeze-drying the eluate, was dissolved in

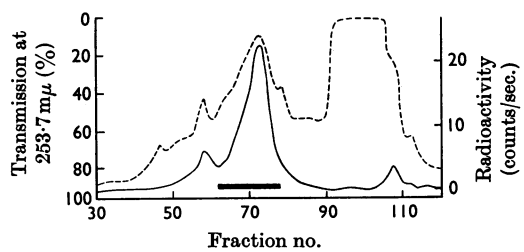


Fig. 1. Gel filtration of the soluble tryptic–chymotryptic peptides from ASPM–GDH (850mg.) through a column (2·3cm.×2·5m.) of Sephadex G-25 (fine grade). The flow rate was 25ml./hr., fractions being collected every 6min.; the solvent was 0·01% thiodiglycol. ----, Percentage transmission at 253·7mμ; ——, radioactivity (counts/sec.); ■, fractions that were further purified.

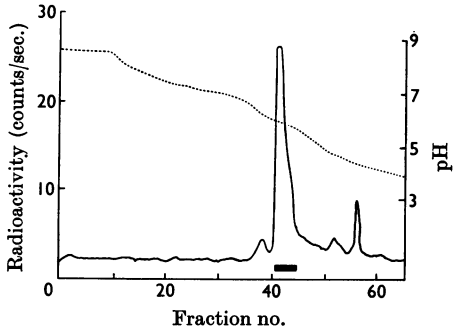


Fig. 2. Purification of peptide ASPM–GDH-TC-1 by chromatography of the radioactive material from the Sephadex column (Fig. 1) on Dowex AG1 (X2). ——, Radioactivity (counts/sec.); ·····, pH of the eluate; ■, fractions that were further purified.

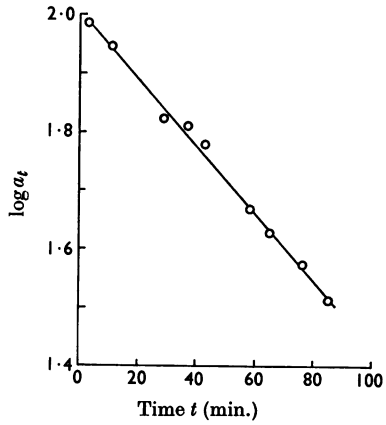


Fig. 3. Kinetics of the loss of enzyme activity of a solution of GDH (5·02mg./ml.) and ASPM (1·94mM) incubated at 25° in 67mM-KH_2PO_4 adjusted to pH7·3 with 5N-NaOH. a_t, residual enzyme activity (%) at time t.

pyridine–acetic acid–water (129:2280:5642, by vol.), pH3·1 (buffer *A*), and was applied to a column (1·1cm.× 45cm.) of Dowex 50 W (X2; 200–400 mesh) that had been equilibrated with the same buffer. The column was then eluted at 12·5ml./hr. with a gradient drawn from a stirred cylindrical vessel A containing buffer *A* (75ml.) connected to a vessel B, of twice the cross-sectional area of vessel A, that contained pyridine–acetic acid–water (129:100:577, by vol.), pH5·0 (150ml.). All the radioactive material applied to the column was eluted as a single peak when the eluate was between pH4·65 and pH4·80 and was recovered by concentrating the eluate to 0·5ml. and filtering it through a column (0·9cm.×80cm.) of Sephadex G-10 equilibrated with 0·2% (v/v) formic acid. The radioactive eluate was freeze-dried, and the residue was designated peptide ASPM–GDH-TC-1.

Isolation of N^{ϵ}*-succin-2-yl-lysine from peptide ASPM–GDH-TC-1.* The peptide (0·2μmole) was heated at 110° in constant-boiling HCl (0·5ml.) for 48hr. in a sealed evacuated tube. The residue after freeze-drying was separated by electrophoresis at pH6·5. Guide strips of the thin-layer plate were sprayed with ninhydrin. A basic band with the same mobility as lysine, a neutral band, and an acidic band that migrated to the anode at a rate of 40% of that at which lysine migrated to the cathode were revealed. The acidic compound was eluted from the unsprayed portion of the plate and was compared with N^{ϵ}-succin-2-yl-lysine.

Hydrolysis of peptide ASPM–GDH-TC-1 with subtilopeptidase. Peptide ASPM–GDH-TC-1 (0·2μmole) was incubated at room temperature for 4hr. in 40mM-NH_4HCO_3 (0·2ml.) containing subtilopeptidase (25μg.). The residue after freeze-drying was applied to Whatman 3MM paper and subjected to electrophoresis at pH6·5 in an apparatus of the Michl (1951) pattern for 1hr. at 55v/cm. Guide strips were developed with ninhydrin. A basic substance migrated 11·6cm. to the cathode and was not radioactive (peptide ASPM–GDH-TC-1-S-1). A neutral substance that was not radioactive was present. An acidic compound migrated 6·6cm. to the anode and was radioactive (peptide ASPM–GDH-TC-1-S-3). The compounds were eluted with dilute ammonia and were recovered by freeze-drying.

RESULTS

The inhibition of GDH by ASPM followed first-order kinetics (Fig. 3) at least down to 30% residual activity. A sample of GDH whose enzymic activity had been 67% inhibited by ASPM had incorporated 0·67 mole of ASPM/52500g. of protein. This incorporation was significantly lower than when the inhibition was at pH6·9, where the results of Pfleiderer *et al.* (1966) predict an incorporation of 0·67 mole of ASPM/45000g. of protein.

Peptide 'maps' of the tryptic peptides prepared from ASPM–GDH showed the presence of a number of radioactively labelled compounds (Fig. 4*b*). The number of these compounds was not decreased by varying the time of the digestion, changing the concentration of trypsin, omitting the acid denaturation, or increasing the temperature. Departure from the described conditions of digestion often left much undigested radioactive material at the origin of the 'map'. However, when labelled protein was digested with both trypsin and chymotrypsin (Fig. 4*a*), the radioactivity of the preparation was found to be associated with only one basic compound (peptide ASPM–GDH-TC-1). Treatment of the whole mixture of tryptic peptides or an isolated radioactive tryptic peptide (peptide ASPM–GDH-T-1) with chymotrypsin resulted in the same basic compound (peptide ASPM–GDH-TC-1) being produced. Peptide 'maps' of the chymotryptic–tryptic peptides derived from GDH inhibited by ASPM at pH6·9 contained a second radioactive compound, which was neutral. Thus the labelling reaction was more specific at pH7·3 than at pH6·9.

Peptides ASPM-GDH-TC-1 and ASPM-GDH-T-1 were isolated by conventional techniques.

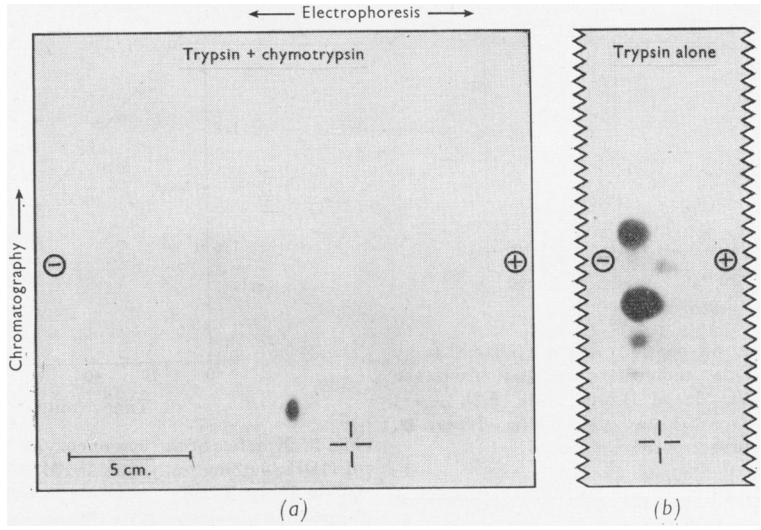


Fig. 4. (*a*) Radioautograph of the chymotryptic–tryptic peptides derived from ASPM–GDH that had been separated as a thin-layer 'map' by the method of Wieland & Georgopoulos (1964). +, Origin; horizontal dimension, electrophoresis for 90min. in water–pyridine–acetic acid (89:10:1, by vol.) at 50v/cm.; vertical dimension, chromatography in pyridine–butane-1-ol–acetic acid–water (40:68:14:25, by vol.). (*b*) Radioautograph of the neutral region of a similar 'map' of the tryptic peptides derived from ASPM–GDH. The conditions were as in (*a*).

Table 1. *Comparison of the succinyl derivatives of amino acids with the unknown amino acid from peptide ASPM–GDH-TC-1*

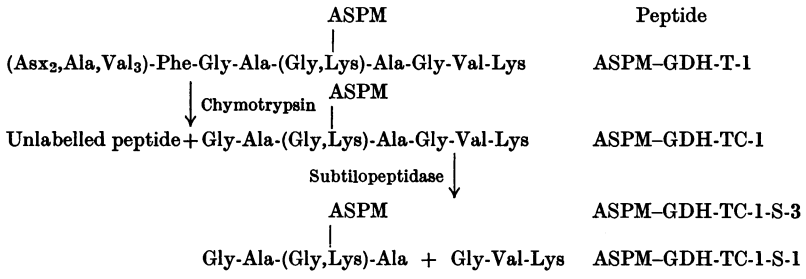
Compound	Position of elution from amino acid analyser	R_F in butan-2-ol–acetic acid–water (2:1:1, by vol.)	Mobility to cathode relative to lysine on electrophoresis in 5% (v/v) formic acid	Mobility to anode relative to the mobility of lysine to cathode on electrophoresis at pH6·5
S-Succin-2-ylcysteine	Before Asp	0·47	0·38	—
N^{ϵ}-Succin-2-yl-lysine	Between Ala and Val	0·19	0·56	0·40
N-Succin-2-ylhistidine	Before Glu	0·22	0·48	0·50
Unknown amino acid in peptide ASPM–GDH-TC-1 after hydrolysis with HCl	Between Ala and Val	0·19	0·56	0·39

Samples of these peptides that had been hydrolysed in 6N-hydrochloric acid did not contain the expected *S*-succin-2-ylcysteine, but did contain a new amino acid that was eluted from the analyser between alanine and valine. Its exact position in this region was dependent on the particular analyser used. The unknown amino acid had the same electrophoretic and chromatographic mobilities as N^{ϵ}-succin-2-yl-lysine (Table 1). The purified peptide ASPM–GDH-TC-1 migrated to the same position in the standard 'map' as did the radioactivity in the mixture of freshly prepared peptides. Peptide ASPM–GDH-TC-1 was finally isolated in a pure state at an overall yield of 10%, and was the same as the radioactive compound present in the initial digest of ASPM–GDH. Since 96% of the radioactivity of ASPM–GDH was applied to the plate that yielded the radioautograph (Fig. 4*a*) a minimum of about 85% of the radioactivity incorporated into GDH is present as ASPM–GDH-TC-1 immediately after the digestion of the protein with chymotrypsin plus trypsin.

The position to which purified peptide ASPM–GDH-T-1 migrated was the same as that to which the minor radioactive component in the freshly prepared tryptic digest of ASPM–GDH migrated. Decomposition occurred both during storage and during chromatographic separations and thus the amount of radioactivity finally isolated as pure peptide ASPM–GDH-T-1 was only 1% of that in the

Table 2. *Amino acid analyses*

	Amino acid composition (relative molar proportions)			
Peptide	ASPM–GDH-T-1	ASPM–GDH-TC-1	ASPM–GDH-TC-1-S-1	ASPM–GDH-TC-1-S-3
Amount analysed (nmoles)	30	70	35	60
No. of analyses	2	6	1	1
Charge at pH 6·5	Neutral	Basic	Basic	Acidic
Amino acid				
Aspartic acid	1·84	—	—	—
Glycine	3·10	2·95	0·92	1·95
Alanine	3·03	1·90	—	1·90
Succinyl-lysine	1·00	1·01	—	1·15
Valine	3·97	0·98	0·97	—
Phenylalanine	1·14	—	—	—
Lysine	0·98	1·15	1·11	—



Scheme 1. Asx, Aspartic acid or asparagine residue.

original ASPM–GDH. The reason for the instability of the radioactive tryptic peptides is not clear. However, if the whole mixture of tryptic peptides, a partially purified preparation, or pure peptide ASPM–GDH-T-1 was treated with chymotrypsin, then all the radioactivity in the preparation was converted into peptide ASPM–GDH-TC-1. Thus, whatever the cause of the instability, it is not due to migration of the radioactive label.

The results of the amino acid analyses of the acid hydrolysates of various peptides are shown in Table 2.

Peptide ASPM–GDH-TC-1-S-1. 'Dansylation' of the peptide and hydrolysis yielded 'dansyl'-glycine. After one Edman degradation, 'dansylation' and hydrolysis, 'dansyl'-valine was detected. Thus the structure is Gly-Val-Lys.

Peptide ASPM–GDH-TC-1-S-3. 'Dansylation' and hydrolysis yielded 'dansyl'-glycine. Hydrazinolysis yielded free alanine. The peptide was radioactive. Thus a partial structure is

ASPM
|
Gly-(Ala,Gly,Lys)-Ala.

Peptide ASPM–GDH-TC-1. Carboxypeptidase B digestion of the peptide yielded free lysine. Submission of the peptide to two cycles of the paper-strip Edman degradation yielded the phenylthiohydantoin derivatives of glycine (cycle 1) and alanine (cycle 2). The peptide was radioactive.

The results are compatible with the derivation of the peptides shown in Scheme 1.

DISCUSSION

The results presented here demonstrate that the modification of a single lysyl residue in an amount of protein corresponding to the peptide chain (Appella & Tomkins, 1966) is sufficient to destroy the catalytic activity of ox liver GDH. This lysyl residue is 'essential', since the modification destroys the catalytic activity of the enzyme. Used in this sense, 'essential' does not imply that the group undergoes reaction during the turnover of the enzyme, although it does not preclude this possibility. There is no reason to suspect that the initial site of ASPM attack is a side chain other than the ϵ-amino group of the lysyl residue on which it is found after proteolytic hydrolysis of the modified protein, especially since experiments under conditions similar to those used here with peptides in which thiol groups were modified with ASPM gave no indication that exchange reactions were possible.

Appella & Tomkins (1966) concluded from their

experiments on the protein chemistry of this enzyme that it was composed of identical polypeptide chains of molecular weight 52000. The isolation of a unique labelled peptide certainly supports this conclusion at least for a sequence of eight residues. One explanation of our inability to devise conditions of tryptic digestion that would yield a single radioactive pentadecapeptide would be that, outside the octapeptide region, there are non-identical regions of the peptide chain. Even to consider this would, however, be premature before the reason for the instability of the labelled tryptic peptides is known.

The reaction of ASPM with a lysyl residue in preference to thiol groups in a protein that contains both types of group was unexpected. *N*-Substituted maleimides are known to react with the ϵ-amino groups of lysines, the ring nitrogen atoms of histidine and proline, and the α-amino groups of peptides and amino acids (Smyth *et al.* 1964; Brewer & Riehm, 1967; Sharpless & Flavin, 1966), but these reactions have half-times of about 2hr. under conditions where the reaction with thiol groups is complete within less than 20sec. (Schnetger, 1966). This is therefore an example of the interactions of the protein thiol groups with other parts of the protein decreasing their reactivity to such an extent that amino groups react most rapidly.

The rate of reaction of the amino group in GDH with ASPM appears to be faster than would be expected for a typical protein amino group. For example, none of the 23 amino groups of lactate dehydrogenase react with ASPM under even more favourable conditions than were used for GDH. Smyth *et al.* (1964) could not detect the reaction of ϵ-amino groups of lysyl peptides with *N*-ethylmaleimide under similar conditions of temperature and pH to those used here. The rate of reaction of the α-amino groups of these peptides was also about one-twentieth of the rate of reaction of ASPM with GDH, and ASPM is less reactive, at least towards thiol groups, than is *N*-ethylmaleimide. The reaction of the ϵ-amino groups of lysine with *N*-ethylmaleimide was observed by Brewer & Riehm (1967) and it is apparent that some of the amino groups of lysozyme and ribonuclease, the two proteins that they studied, are as reactive as the 'essential' amino group of GDH. The sequence of amino acids around the essential ϵ-amino group of GDH consists of non-polar amino acid residues. Such a non-polar region would diminish the tendency of an amino group to take up protons and might result in the pK of the lysyl residue being abnormally low.

Both Anderson *et al.* (1966) and Colman & Frieden (1966*a*,*b*) have caused GDH to react with reagents that appeared to modify lysyl residues. Neither of these groups of workers could, however, determine whether the results of these modifications on the ability of the enzyme to catalyse its characteristic reaction, to aggregate at high protein concentrations or to respond to effectors such as GTP were due to the modification of a unique group, or to random reaction of amino groups over the whole GDH molecule. The ability to modify a unique ϵ-amino group of GDH will allow a distinction to be made between these possibilities.

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