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Ox liver glutamate dehydrogenase

The use of chemical modification to study the relationship between catalytic sites for different amino acid substrates and the question of kinetic non-equivalence of the subunits

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1. The effect of pyridoxal 5′-phosphate on the activity of ox liver glutamate dehydrogenase towards different amino acid substrates was investigated. 2. Both alanine and glutamate activities decreased steadily in the presence of pyridoxal 5′-phosphate. 3. The alanine/glutamate activity ratio increased as a function of inactivation by pyridoxal 5′-phosphate, indicating that glutamate activity is lost more rapidly than alanine activity. 4. A mixture of NADH, GTP and 2-oxoglutarate completely protected the alanine and glutamate activities against inactivation by pyridoxal 5′-phosphate. 5. The activity of glutamate dehydrogenase towards glutamate and leucine decreased steadily in a constant ratio in the presence of pyridoxal 5′-phosphate. 6. The effect of leucine on the alanine and glutamate activities as a function of inactivation by pyridoxal 5′-phosphate was studied. 7. The results are interpreted to suggest that the subunits of glutamate dehydrogenase hexamer are kinetically non-equivalent with regard to activity towards the two monocarboxylic amino acids as well as glutamate, and that all three substrates share the same active centre. However, leucine is also able to bind at a separate regulatory site.

Ox liver GDH (EC 1.4.1.3) is known to catalyse the oxidative deamination of certain monocarboxylic amino acids as well as L-glutamate (Struck & Sizer, 1960). Prough *et al.* (1972) have used u.v. difference spectroscopy to demonstrate the formation of a GDH–L-leucine complex. Various authors have reported the activatory action of monocarboxylic amino acids on GDH (Yamaguchi, 1971; Bitensky *et al.*, 1965*b*; Hershko & Kindler, 1966; Yielding & Tomkins, 1961; Markau & Steinhübel, 1972), in particular that of L-leucine, L-alanine, L-methionine and L-norvaline. According to Yielding & Tomkins (1961), L-leucine stimulates the oxidation of glutamate rather than vice versa, since leucine stimulates equally well the reduction of 2-oxoglutarate. Parallels are drawn between the leucine effects and NAD^+ and ADP effects. These authors postulate that leucine has the same site of action as ADP but a different one to glutamate, since activation rather than inhibition was observed, and they state that the leucine and alanine sites are also separate.

Abbreviation used: GDH, glutamate dehydrogenase.

Hershko & Kindler (1966) have reported, however, that the purine nucleotides and the monocarboxylic amino acids bind to distinct group-specific allosteric sites, since different combinations of chemically unrelated activators (e.g. L-leucine and ADP) enhance the activity in a synergistic manner.

Work by Frieden (1963), Tomkins *et al.* (1961, 1965) and Bitensky *et al.* (1965*a*,*b*) led to the postulate that the enzyme existed in two conformations, designated ‘monomer x’ and ‘monomer y’, which were in equilibrium with each other. According to this scheme, monomer x, with an M_r of 250000, associates to produce the higher-molecular-mass polymer, with an M_r of 1000000, as its concentration is raised, and is enzymically active for glutamate; monomer y is active for alanine and does not undergo association. However, the naming of these forms is confusing in the light of current knowledge of the quaternary structure, according to which the enzyme is a hexamer with a subunit M_r of 56000. The hexamer undergoes further linear aggregation, but the M_r of 10^6 does not correspond to a single clearly defined molecular form of the enzyme. However, although

it has now been shown that there is no direct causal connection between association and enzymic activity towards glutamate (Fisher *et al.*, 1962; Iwatsubo & Pantaloni, 1967; Reisler & Eisenberg, 1972; Josephs *et al.*, 1972), this does not invalidate the basic idea of two conformations. Thus the finding by Tomkins *et al.* (1965) that diethylstilboestrol, ADP and GTP have reciprocal effects on the monocarboxylic amino acid and glutamate activities of GDH can still plausibly be interpreted in terms of perturbation of the equilibrium betweem two forms with optimal glutamate activity and optimal monocarboxylic amino acid activity respectively. Similar reciprocal effects of mercurials on alanine and glutamate activities have been reported by Bitensky *et al.* (1965*a*).

Pyridoxal 5′-phosphate has been found by various workers to decrease the activity of GDH towards glutamate/2-oxoglutarate (Piszkiewicz & Smith, 1971; Goldin & Frieden, 1971; Brown *et al.*, 1973). Chen & Engel (1975) showed unequivocally that the chemical modification caused total inactivation of the affected subunits with glutamate as the substrate. To our knowledge, however, the effect of pyridoxal 5′-phosphate on activity towards other amino acid substrates has not previously been investigated. This appeared to be a promising line of inquiry, both in order to resolve some of the uncertainties concerning the relationship between the sites for activity towards glutamate, alanine and leucine, and to explore the possibly related question of whether the six subunits within a GDH hexamer are strictly equivalent in their catalytic behaviour. The results reported in the present paper indicate, in contrast with earlier suggestions (Yielding & Tomkins, 1961), that both leucine and alanine share the same active site as glutamate. It would appear, however, that leucine also has a separate regulatory site, and that, within the hexamer, some subunits are relatively more active towards monocarboxylic acids than are others.

Materials and methods

Materials

Ox liver GDH was obtained from Biozyme Laboratories, Blaenavon, Gwent, U.K., as a crystalline suspension in $(NH_4)_2SO_4$ solution and stored at 4°C. For each experiment, a sample of the suspension was centrifuged, resuspended in 0.1 M-potassium phosphate buffer, pH 7.6, containing 10 μM-EDTA and dialysed against the same buffer. The GDH solution was clarified by centrifugation and the concentration of the enzyme calculated from A_{278} measurements by using the specific absorption coefficient of 0.93 litre·g^{-1}·cm^{-1} (Egan & Dalziel, 1971). NAD^+ used in these experiments was a product (grade II) of Boehringer Corporation, Lewes, East Sussex, U.K., and was routinely repurified essentially according to the method of Dickinson & Engel (1977) by chromatography on DEAE-Sephadex A-25 (Pharmacia, Hounslow, Middx., U.K.) followed by desalting on Bio-Gel P2 (Bio-Rad Laboratories, Bromley, Kent, U.K.).

Conditions for inactivation of GDH

GDH incubations (1 mg/ml) contained 0.13–1.8 mM-pyridoxal 5′-phosphate in 0.1 M-potassium phosphate buffer, pH 7.6, and were equilibrated at 25°C in small test tubes wrapped in metal foil to prevent breakdown of pyridoxal 5′-phosphate. The inactivation was initiated by the addition of pyridoxal 5′-phosphate and was followed by measuring enzyme activity with glutamate as the substrate. Samples (10 μl) were withdrawn at timed intervals and assayed in 4 ml assay mixtures, also pre-equilibrated at 25°C, containing 240 μM-NAD^+ and 40 mM-glutamate in 0.1 M-potassium phosphate buffer, pH 7.6. The rate of reaction was measured by the use of a recording fluorimeter as described by Engel & Dalziel (1969). GDH was inactivated to different extents by stopping the reaction at different times by the addition of 100 μl of ice-cold 100 mM-$NaBH_4$/ml of incubation mixture. The reduction was left to proceed in the dark at 4°C for 30 min, after which time the reduced samples were dialysed against the same buffer in the dark at 4°C overnight. The dialysed samples were clarified by centrifugation, and the activity towards glutamate and alanine was measured in triplicate.

Results and discussion

Fig. 1 shows the effect of pyridoxal 5′-phosphate on GDH activities towards alanine and glutamate. The residual activity was expressed as a percentage of the activity in a control sample, i.e. unmodified GDH that had undergone the same treatment as the modified GDH except for the omission of pyridoxal 5′-phosphate. Fig. 1 shows plots of percentage residual alanine activity versus percentage residual glutamate activity, and of the alanine/glutamate activity ratio versus percentage residual glutamate activity. The dashed line is based on the assumption that all six GDH subunits are identical in every respect. According to this, both alanine and glutamate activities would be expected to decrease in exact parallel with increasing extent of inactivation. The dotted horizontal line shows the expected result on the alternative assumption that alanine and glutamate sites are entirely independent and that pyridoxal 5′-phosphate destroys activity only at glutamate sites. Fig. 1 shows clearly that neither of these

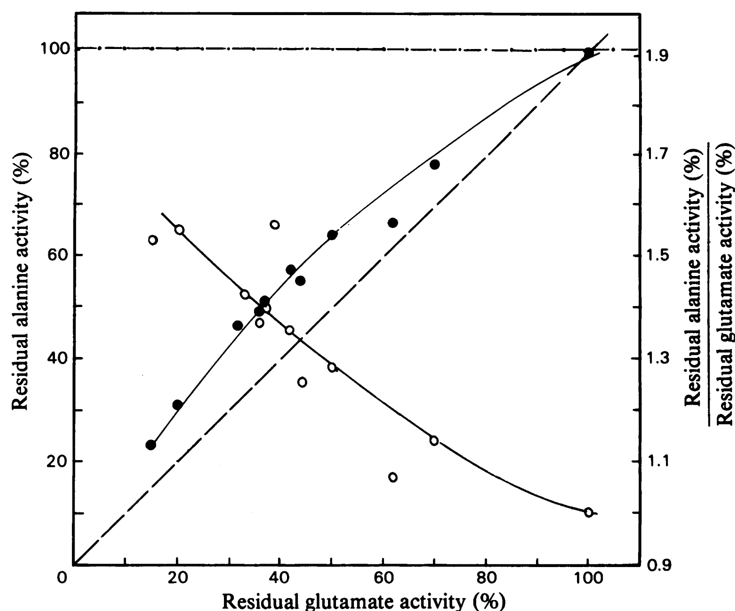

Fig. 1. *Effect of pyridoxal 5′-phosphate on ox liver GDH activities towards alanine and glutamate*
Various enzyme incubations were inactivated with 0.13–1.8mM-pyridoxal 5′-phosphate and reduced with 100mM-$NaBH_4$ as described in the Materials and methods section. The alanine assay mixture contained 240mM-NAD^+ and 100mM-alanine. A 50μg portion of enzyme was used for assay of catalytic activity. Percentage residual alanine activity (●) and the alanine/glutamate activity ratio (○) are both plotted (vertical axis) against percentage residual glutamate activity (horizontal axis). The dashed and the dotted lines are discussed in the text.

predictions is fulfilled. The alanine/glutamate activity ratio increases with the extent of inactivation, but nevertheless the alanine activity, as well as the glutamate activity, decreases steadily. Moreover, a mixture of 2.1mM-NADH, 1mM-GTP and 1mM-2-oxoglutarate completely protected both the glutamate and the alanine activities against inactivation by pyridoxal 5′-phosphate.

Pyridoxal 5′-phosphate is known to modify two residues in GDH, lysine-126 (Anderson *et al.*, 1966; Piszkiewicz *et al.*, 1970) and lysine-333 (Talbot *et al.*, 1977). Lysine-126 has been shown to be essential for GDH activity towards glutamate (Chen & Engel, 1975; Talbot *et al.*, 1977), whereas lysine-333 appears not to be essential for activity towards glutamate. It seemed possible, however, that alanine might be oxidized at a separate site, which perhaps involved lysine-333. It is known that the combination of NADH, GTP and 2-oxoglutarate protects lysine-126 completely but does not prevent modification of lysine-333 (Talbot *et al.*, 1977). Thus the present results indicate that lysine-126 is essential for the activity towards alanine as well as that towards glutamate, and strongly suggest that both substrates have the same active centre. However, the fact that glutamate activity is lost more rapidly than alanine activity indicates that, although the six identical subunits are all expected to contribute to both activities, some subunits are relatively more active towards alanine than others. There is thus a degree of non-equivalence of the six subunits within the same hexamer.

The above conclusion is further substantiated by the results in Fig. 2, which shows the effect of 10mM-L-leucine on the residual alanine and glutamine activities of GDH as a function of inactivation by pyridoxal 5′-phosphate. As can be seen, leucine has opposite effects on activities toward alanine (inhibition) and glutamate (activation), similar to those reported by other authors (Frieden, 1963; Tomkins *et al.*, 1965; Bitensky *et al.*, 1965*a*). Clearly, also, the extent of enhancement of glutamate activity by leucine increases as the enzyme is progressively inactivated by pyridoxal 5′-phosphate. The activation increases from 30% in fully active GDH to approx. 70% with GDH retaining only 30% of its activity in the standard glutamate assay. The alanine activity, on the other hand, is inhibited by leucine to the same extent (approx. 55%) irrespective of the extent of inactivation by pyridoxal 5′-phosphate.

If it is assumed that all subunits of GDH behave identically, then the glutamate activity should be stimulated to the same extent independent of the degree of inactivation. Since GDH partially

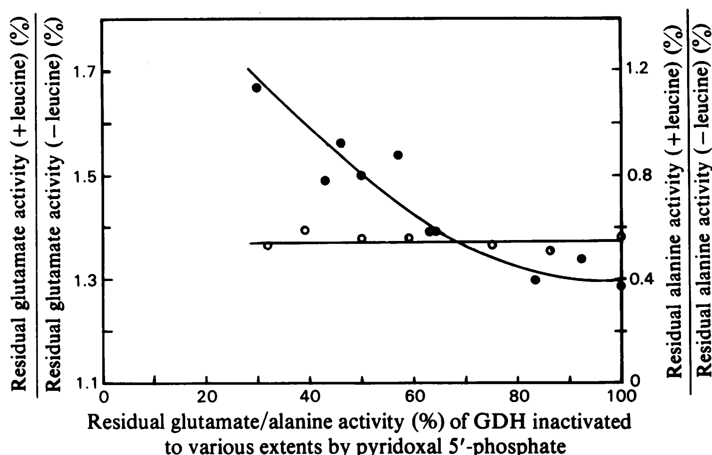

Fig. 2. *Effect of L-leucine on the activities of ox liver GDH towards alanine and glutamate as a function of inactivation by pyridoxal 5′-phosphate*

Various GDH incubations were inactivated with 0.13mM-pyridoxal 5′-phosphate and reduced with 100mM-$NaBH_4$ as described in the Materials and methods section. After dialysis of reduced GDH samples, both glutamate and alanine activities were measured in triplicate in the presence and in the absence of 10mM-L-leucine in 4ml assay mixtures. The concentrations of coenzyme and substrates were the same as those indicated in Fig. 1 legend. The ratio of activities with and without leucine present are plotted for glutamate (●) and alanine (○) as substrate against percentage residual activity measured in the absence of leucine for each substrate.

inactivated by pyridoxal 5′-phosphate undergoes greater stimulation by leucine, it appears that the subunits modified last within the hexamer show greater sensitivity to this effector. However, it is difficult to establish whether this results from an intrinsic positional non-equivalence of the subunits or whether modification of some of the subunits induces changes in the remaining active subunits, altering their kinetic behaviour.

The fact that leucine activates glutamate activity even more strongly when most of the sites for glutamate oxidation have been rendered inactive by pyridoxal 5′-phosphate strongly suggests that leucine binds to a separate regulatory site. If leucine were only able to bind at the glutamate sites, one might expect the dominant effect in enzyme molecules with only one or two functional catalytic sites remaining to be a simple competition, resulting in inhibition.

However, leucine also acts as an alternative amino acid substrate for GDH (Struck & Sizer, 1960; Markau & Steinhübel, 1972), as indicated by the results presented in Fig. 3 and Table 1. Fig. 3 shows time courses of inactivation of leucine and glutamate activities by pyridoxal 5′-phosphate; the two activities declined in constant ratio. This result suggests that, for its oxidation, leucine indeed has the same active centre as glutamate. The constant ratio of activities contrasts with the results shown in Fig. 1. This is somewhat surprising, since the two monocarboxylic amino acids might be expec-

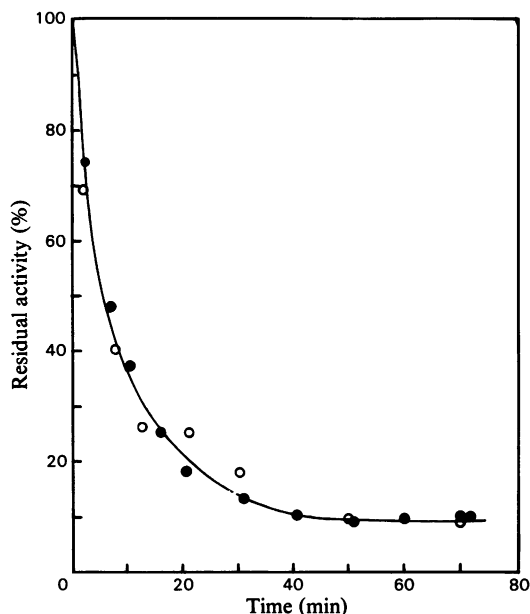

Fig. 3. *Time courses of inactivation of ox liver GDH activities towards leucine and glutamate*

The inactivation (see the Materials and methods section) was carried out in the presence of 0.45mM-pyridoxal 5′-phosphate. The assay mixtures contained 240μM-NAD^+ and either 108mM-leucine or 40mM-glutamate. A 10μg portion and a 50μg portion of enzyme were used for assay of glutamate (●) and leucine (○) activity respectively.

Table 1. *K_m and $V_{max.}$ parameters derived from Lineweaver–Burk plots for the oxidation of* L*-leucine,* L*-alanine and* L*-glutamate by ox liver GDH at pH 7.6*

Activity measurements were carried out in 4ml assay mixtures in 0.1 M-potassium phosphate buffer, pH 7.6, as described in the Materials and methods section. The NAD^+ concentration was 240 μM and the concentration of L-glutamate was varied in the range 5–40 mM. The final enzyme concentration was 1 μg/4 ml. In the case of L-leucine and L-alanine, the concentration ranges were 14.7–100 mM and 10–80 mM respectively. The final enzyme concentration in both cases was 50 μg/4 ml. The K_m and $V_{max.}$ parameters (means ± S.E.M.) were evaluated by using a non-linear regression program, written for the Wang desk-top computer by Dr. J. Kinderlerer (unpublished work).

Substrate	$V_{max.}$ (μmol/min per μg)	K_m (mM)
L-Leucine	$9.37 \times 10^{-4} \pm 0.45 \times 10^{-4}$	111 ± 9
L-Alanine	0.29 ± 0.02	404 ± 3
L-Glutamate	0.77 ± 0.003	1.1 ± 0.04

ted to behave similarly in this case, especially in view of the similarities observed by other workers (Yielding & Tomkins, 1961; Tomkins *et al.*, 1965; Hershko & Kindler, 1966; Yamaguchi, 1971).

Markau & Steinhübel (1972) reported competitive inhibition by leucine (100 mM) of the oxidative deamination of glutamate. This supports the suggestion of a common site. On the other hand, Yielding & Tomkins (1961) proposed that, because leucine (12 mM) stimulated glutamate activity rather than inhibiting it, leucine could not have the same site as glutamate. Taking into account the results of both Fig. 2 and Fig. 3, it appears that the resolution of this apparent contradiction lies in the ability of leucine to bind both at the glutamate (catalytic) site and also at a separate regulatory site.

The inhibitory effect of leucine on alanine activity may not be exclusively due to the binding of leucine to the regulatory site. It could also be explained by competition between the two substrates at the active site. Table 1 shows the K_m and $V_{max.}$ parameters derived from Lineweaver–Burk plots for the two monocarboxylic amino acids at pH 7.6. Experimental details are given in the legend. As can be seen, the K_m values for both monocarboxylic amino acids are very large compared with that for glutamate at pH 7.6. At this pH, the K_m for L-alanine is 4 times larger than that for L-leucine, but the $V_{max.}$ for L-alanine is 286 times larger than that for L-leucine.

It thus seems quite likely that leucine can displace alanine from the active site but that, having done so, it gives a much lower rate of reaction. In the case of glutamate, such inhibition would be negligible, if, as seems likely, this substrate binds much more tightly than either leucine or alanine, and, in any case, any inhibition would be further masked by the stimulatory effect that is associated with binding at the leucine regulatory site.

In connection with the view expressed above that the six subunits of the GDH hexamer are not kinetically equivalent, a number of earlier observations are relevant. Rasool *et al.* (1976) reported that the time courses for inactivation of GDH 3,6-diamino-1-chlorohexan-2-one ('L-glutamyl α-chloromethylketone') were biphasic. The results were interpreted to suggest the involvement of 'negatively co-operative' interactions in the reactivity of lysine-126. They argued that the first subunit that takes part in catalysis makes the largest, and the last the smallest, contribution to the overall catalysis, and emphasized that three of the six subunits may contribute as much as 80% of the total activity of GDH towards glutamate. The idea of the non-equivalence of GDH subunits is further supported by the work of Smith & Bell (1982), who have explained the phenomenon of hysteresis in GDH by a model based on reciprocating subunits. Hysteresis is postulated to result from product (NAD^+) accumulation resulting in a half-of-the-sites activation of reductive amination. Bell & Dalziel (1973), 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 that differ in intrinsic affinity for the coenzyme. Freedman & Radda (1969), Coffee *et al.* (1971) and Goldin & Frieden (1971) have also observed apparent negative co-operativity in the modification of lysine-428 by trinitrobenzenesulphonate. It was found that three of the six subunits were modified exclusively at lysine-425 and the other three exclusively at lysine-428. Further, Talbot *et al.* (1977) found that pyridoxal 5'-phosphate binding to GDH follows biphasic kinetics and that 92% inactivation had occurred when half of the subunits had been modified. Pranab *et al.* (1975) have also shown that 5'-fluorosulphonylbenzoyl-adenosine reacts with the second inhibitory NADPH site and that the interaction of 0.5 mol of the inhibitor/mol of peptide chain abolishes the NADPH inhibition.

Our own studies reported in the present paper suggest that the six subunits are indeed kinetically non-equivalent and that this non-equivalence extends to differences in substrate specificity. It is also clear from the present work that there are not separate catalytic sites for the oxidation of monocarboxylic amino acids. They are oxidized at the same site as glutamate. Leucine, however, is also able to bind at a separate regulatory site.

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