KINETIC MEASUREMENTS WITH MONOCARBOXYLIC ACIDS AS SUBSTRATES AND EFFECTORS OF GLUTAMATE DEHYDROGENASE*

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1. Introduction

Some monocarboxylic amino acids as e.g. leucine are known to be substrates of the bovine liver glutamate dehydrogenase (1.4.1.3) at pH values above 8 [2], whereas at lower pH values they only activate the oxidation of glutamate [3]. A simple model of the active center of the enzyme would require only one binding subsite for the α-amino acid configuration of both mono- and dicarboxylic amino acids and would predict competitive inhibition rather than activation. The present paper shows that four kinetic effects of the leucine must be differentiated which in sum cause activation at high glutamate concentration and inhibition at lower ones. One of these effects is a competitive inhibition of glutamate by leucine but also the three others can be explained in principle by one single leucine binding site which is identical with that for glutamate. In addition it is shown that GTP which is known to inhibit the glutamate oxidation [4] and to activate the oxidation of monocarboxylic acids [5], under conditions of high enzyme turnover is also able to inhibit this second reaction.

2. Experimental

Glutamate dehydrogenase (GluDH) and the nucleotides were obtained from Boehringer (Mannheim) as crystalline suspensions. The A_{280}/A_{260} ratio of the enzyme was ≥ 1.95 in phosphate buffer, concentration determinations were based on $\epsilon_{280} = 0.97$. The other chemicals were purchased from Merck (Darmstadt), R.G. or "for biochemistry purpose" with the exception of 4-methylvalerate which was only of "for synthesis" purity. The equipment for the activity measurements and the extrapolation procedure for initial velocities have been described earlier [6].

The ultracentrifuge runs were performed in a Beckman Model E analytical centrifuge with photoelectric scanner equipment. Six-channel cells were used in a six-hole rotor. One substance compartment of each cell contained only the amino acids in the buffer, one other the same mixture plus NADH and in the third NADH and enzyme were added. In this way an optimal calibration of each experiment is guaranteed. The optical density of the NADH was measured at 340 nm in the upper part of the cell after total sedimentation of the protein (40,000 rpm, ca. 30 min).

3. Results and discussion

Fig. 1A shows a Lineweaver–Burk plot of the initial velocities against glutamate concentration in the presence of different leucine concentrations. The control shows an upward curvature at the highest concentrations indicating substrate self-inhibition [7, 8]. At lower concentrations an opposite nonlinearity is observed which has been described recently [7], but which here consists of at least one defined discontinuity (one other is indicated by the measurements at the lowest concentration in connection with less accurate points outside the plot). Thus there exists an obvious correspondence of these results to the dis-

*Studies on glutamate dehydrogenase, part XIII; part XII, see [1].

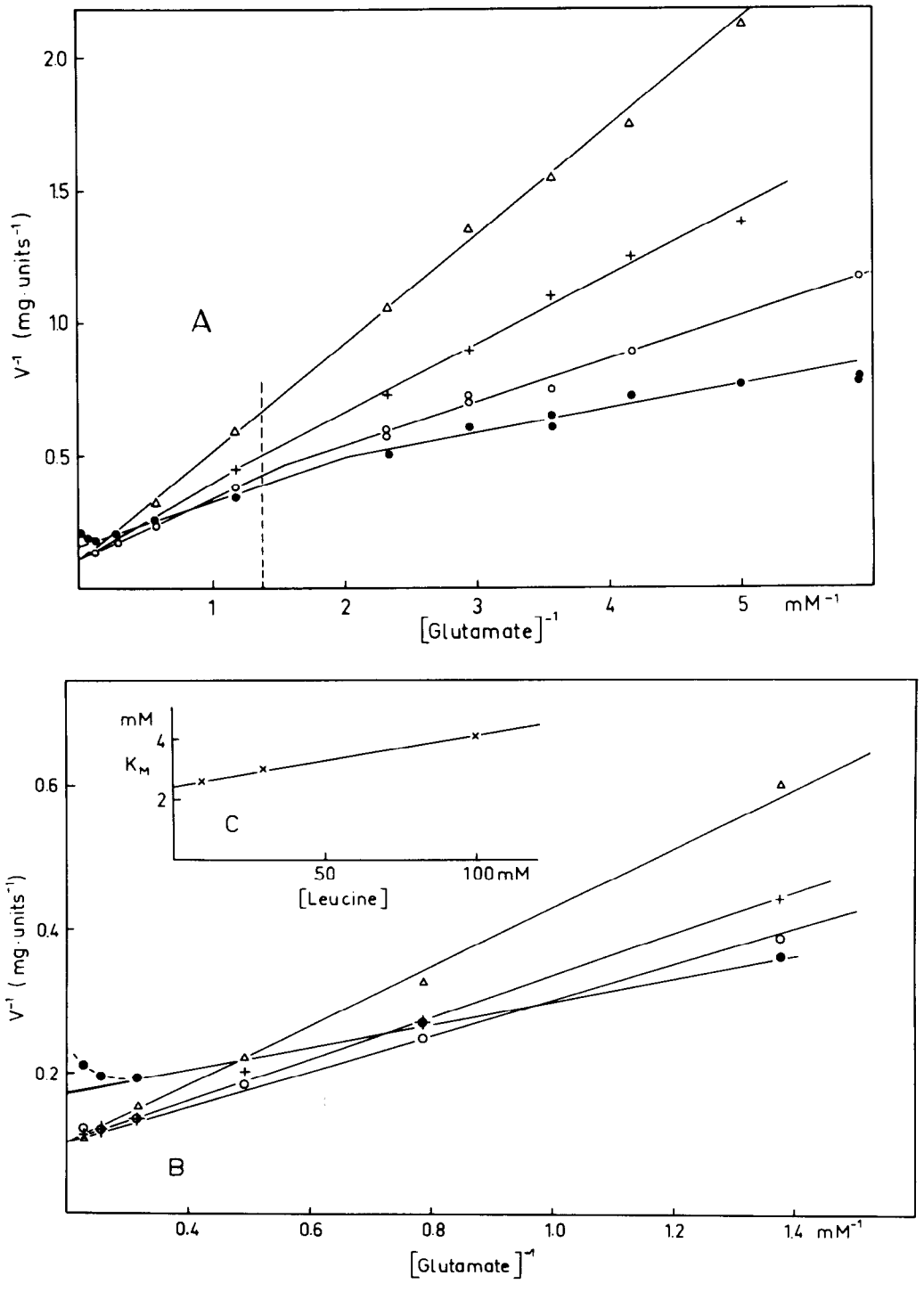

Fig. 1. A) Lineweaver–Burk plot of initial velocities of glutamate oxidation versus glutamate concentration in the presence of several concentrations of leucine. Measurements were performed in 0.067 M sodium phosphate buffer at pH 7.6 and 20° with 1.4 mM NAD^+ and 2.5 μg/ml GluDH. • Control without leucine; ○ with 10 mM; + 30 mM, and △ 100 mM leucine. B) Magnification of the high concentration part of fig. 1A left of the dotted line with some additional points. C) (insert): Secondary plot of the K_m values from fig. 1B versus leucine concentration.

Table 1

Dependence of leucine activation on leucine concentration at the beginning of the glutamate self-inhibition (8.5 mM) with 1.4 mM NAD^+ and 2.5 μg/ml GluDH.

Leucine (mM)	Relative activation v/v_o	% of the saturating value
10	1.44	
5	1.45	100
2.5	1.33	68
1	1.23	43
.5	1.16	24

Conditions as in fig. 1.

Table 2

The relative concentrations of NADH in the centrifugation supernatant of mixtures with GluDH and amino acids in % of the starting NADH concentration (50 μM).

5 mg/ml GluDH	25 %
+ Glu	4.5%
+ Leu	28 %
+Glu + Leu	11 %

Concentrations of glutamate and leucine were 20 mM and 100 mM.

continuities which have been described for the Lineweaver–Burk plot with NAD^+ as variable [8]. In the presence of leucine the substrate self-inhibition disappears, the suppression being practically complete with 30 mM leucine. Also the bend of the curves becomes less noticable and is shifted to higher glutamate concentrations, so that with 100 mM leucine no deviation from a straight line is observed.

The high concentration parts of the curves (fig. 1B) show within the limit of experimental error the same V_{max}, indicating competitive inhibition. From a secondary plot of the apparent Michaelis constants against leucine concentration (fig. 1C) an inhibition constant of 130 mM for leucine and a theoretical limiting glutamate Michaelis constant for disappearing competitive inhibition (on the assumption that other leucine effects still remain) can be obtained by extrapolation. This value of 2.4 mM for K_m of glutamate is much larger than that without leucine, which from the linear part of the high concentration section can be estimated to be 0.95 mM. Obviously this increase of the glutamate Michaelis constant occurs at much lower leucine concentrations than those which are necessary to observe the competitive inhibition. Simultaneously the V_{max} value increases from ca. 5.9 Units $\cdot mg^{-1}$ ($\mu moles/min^{-1} \cdot mg^{-1}$) linearily extrapolated from the high concentration part of the control, to 10 Units $\cdot mg^{-1}$. In order to obtain a characteristic leucine concentration for this effect, the leucine activation was measured just before the beginning of the substrate inhibition (8.5 mM glutamate) for low leucine concentrations. Table 1 shows the results and allows an interpolation to a half-effect concentration of 1.5 mM.

In total we have to deal with four different leucine effects: i) The suppression of the substrate self-inhibition is explained most easily. Stopped-flow measurements have indicated the existence of an abortive enzyme–glutamate–NADPH complex with very slowly dissociating NADPH [9] and a similar complex has to be assumed also with NAD to explain the substrate inhibition. If leucine competes with the glutamate in this complex, the corresponding leucine complex is formed. In contrast to the dicarboxylic acids which induce a strong tightening of the coenzyme binding [10], the monocarboxylic acids seem not to exhibit this effect. Thus the leucine abortive complex dissociates much faster and the substrate inhibition is removed since the release of NADH at high concentrations of substrate and coenzyme is the rate limiting step [11]. Because of the very low NADH concentrations in the tests there does not exist a rapid equilibrium between leucine and glutamate and relatively small concentrations of leucine can induce a significant effect. To show the possibility of such a mechanism more directly, the concentration of NADH was determined in the centrifugation supernatant of a mixture with enzyme in the presence of glutamate, leucine or both simultaneously. Table 2 shows that glutamate enhances the binding, whereas leucine does not and in fact there is even a small reduction of NADH binding, which could be due to unspecific ionic strength effects. In the mixture of glutamate and leucine the binding of NADH is markedly lower than with glutamate alone which indicates the possible competition between the two amino acids in the abortive complex.

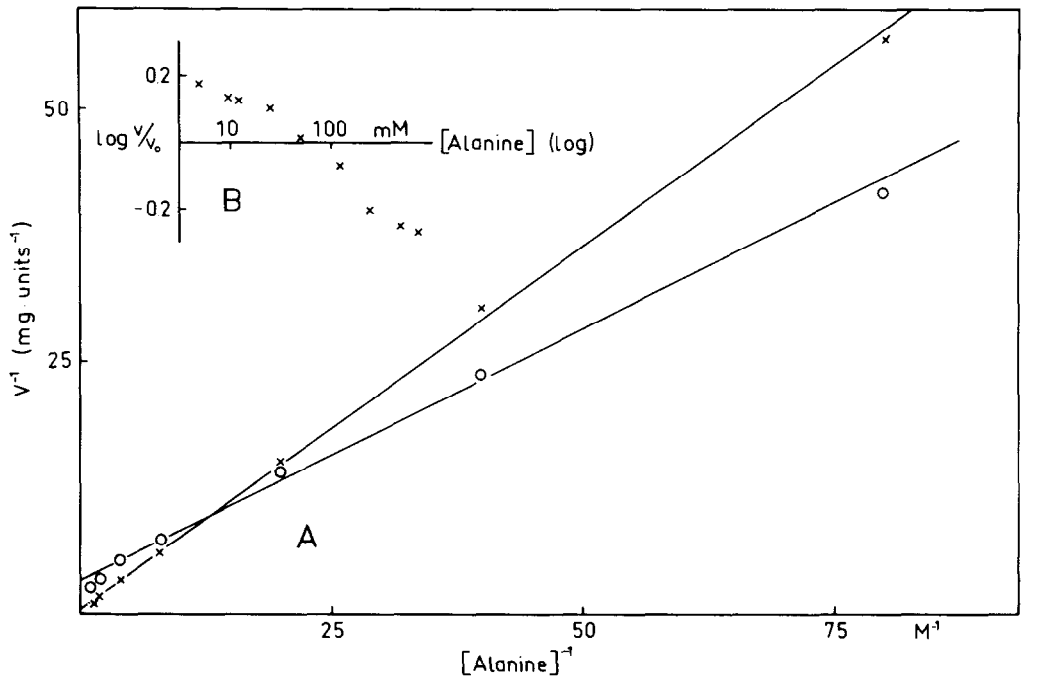

Fig. 2. A) Lineweaver–Burk plot of initial velocities of alanine oxidation versus the alanine concentration in the absence (×) and presence (○) of GTP. Experiments were carried out in 0.065 M glycine buffer with 0.2 M NaCl at pH 9.8 and 20° with 3.6 mM NAD and 0.37 mg/ml GluDH. B) Dependence of the relative effect of GTP on the alanine concentration in a concentration range larger than in fig. 2A. v_0 is the initial velocity in the absence of GTP, v that in the presence of GTP.

The leucine concentration must be higher in this experiment than in kinetic measurements since here a real equilibrium is established.

ii) The competitive inhibition of leucine shows that in all probability the same binding site of the enzyme is used by both substrates. Control experiments with glycine showed no significant inhibition so that an unspecific action of amino acids can be excluded. The very large value for the inhibition constant leads to the conclusion that this competition occurs in the ternary complex with NAD^+, where by reciprocity laws the binding of glutamate must be much stronger than in the binary complex so that the competitive situation for the leucine is less favorable. (The direct competition in the binary complex is discussed below.) The answer to the question of why leucine is not oxidized to a measurable extent in spite of the postulated formation of a reactive ternary complex, can at the moment only be speculative. Maybe under these conditions the dissociation velocity of this complex is so high that the rapid equilibrium mechanism is not valid.

One of the most important objections to a close similarity between the reactions with mono- and dicarboxylic acids, respectively, is the fact that GTP favours the conversion of monocarboxylic acids whereas the oxidation of glutamate is inhibited, so that qualitative differences between the two types of reaction could be postulated. But it has been concluded from stopped-flow measurements that GTP only reduces the velocity of the release of NADH [11] (also strong enhancement of the NADH binding is observed with GTP [12]), whereas the formation of the primary reactive complex is accelerated. Since the second effect can be understood by a tightening of the NAD^+ binding, the simplest model for the GTP effect is a general enhancement of the coenzyme binding. The assumption of an allosteric transformation of the enzyme under the combined influence of GTP and NADH then is not necessary for the explanation of the kinetic effects, especially since in the forward reaction the NADH concentration remains small. Thus, whether GTP is an activator or an inhibitor should only depend on the total turnover of the enzyme and one can hope to find conditions where it also inhibits the oxidation

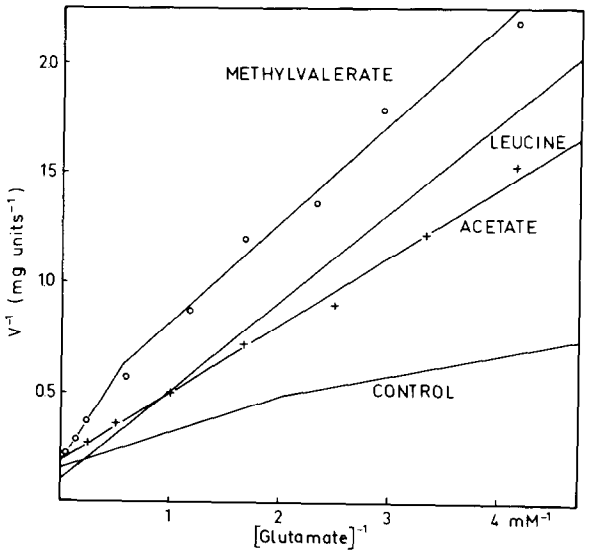

Fig. 3. Lineweaver–Burk plot of glutamate oxidation with different monocarboxylic acids as effectors. Experimental conditions are the same as in fig. 1, the concentration of all effectors was 100 mM. The curves without measured points are taken from fig. 1.

of monocarboxylic acids. For these experiments the tests were performed at the pH optimum (9.8) and first the best of these substrates, norvaline, was used. Results showed that in this case GTP is a strong inhibitor and that it is very difficult to chose substrate and coenzyme concentrations so that activation occurs. This was easier with the poorer substrate alanine. Fig. 2B shows a crossover from activation to inhibition with increasing alanine concentrations. Fig 2A shows a Lineweaver–Burk plot of a part of these data. Without GTP a rather high V_{max} of 2 units is found, indicating that the very large Michaelis constant (1.8 M) is mainly responsible for the slow reaction of alanine. (These experiments show strong product, presumably NADH-inhibition, so that the good extrapolation to the initial velocity is important). GTP causes a decrease of V_{max} as with dicarboxylic acids and its activating action at low substrate concentrations is caused only by a more than tenfold decrease of the Michaelis constant. This could be explained by an indirect action involving an increase in the concentration of the binary enzyme–NAD^+ complex, but a direct interaction between alanine and GTP cannot be excluded for the explanation of the apparent substrate activation at the highest alanine concentrations. These experiments show only quantitative differences between the oxidation of mono- and dicarboxylic amino acids, connected with the poorer binding of the first and the absence of positive cooperativity with the coenzymes.

A further question which arose is why NH_4^+ is a much stronger competitive inhibitor to glutamate [13] than leucine. The amino group seems to give only a small contribution to the total binding energy of the amino acids (since glutarate, for example, is a strong competitive inhibitor [14]), on the other hand it must be responsible for correct orientation of the substrate. This orientation can be hindered by NH_4^+. For this effect the binding energy of NH_4^+ must be compared only with the orientation energy, whereas the binding energy of leucine must be compared to the total binding energy of glutamate. If this model is correct, the leucine analogue without amino group (4-methyl-valerate) should also be a competitive inhibitor. Fig. 3 shows that it is even a stronger inhibitor, but in this case a less specific (partially an ionic strength) effect has to be subtracted. Results indicate that, in contrast to glycine, acetate is inhibitory, indicating a general effect of fatty acids without internal charge compensation. The remaining "specific" effect of methylvalerate is comparable with that of leucine. In addition to the bend in the curve which is not yet understood, the main difference is the absence of a significant influence on V_{max}, and the small decrease in the case of the V_{max} seems to be caused by the unspecific effect.

iii) The third effect which must be discussed is the increase of the glutamate Michaelis constant at relatively low leucine concentrations. An explanation similar to the corresponding ADP effect [6] may obtain, for in a random ordered reaction scheme the pathway beginning with the binding of glutamate is suppressed by low leucine concentrations. Because in this latter case the competition of glutamate is not enhanced by the coenzyme. As in the case of ADP fluorometric measurements with very low NAD^+ concentrations should give a direct proof for this hypothesis.

iv) Most difficult is the explanation of the increase in the linearly extrapolated V_{max} without the assumption of a special regulatory leucine binding site. But one could imagine that a competition with oxoglutarate in the final ternary enzyme–oxoglutarate–NADH

complex could accelerate the release of NADH at high enzyme turnover.

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