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EFFECT OF 2-OXOGLUTARATE ON THE CATALYTIC ACTIVITY AND STABILITY OF GLUTAMATE DEHYDROGENASE

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SUMMARY

1. ATP-induced inhibition of the NADH oxidation reaction catalyzed by glutamate dehydrogenase (L-glutamate:NAD(P) oxidoreductase (deaminating), EC 1.4.1.3) is counteracted by high concentrations of 2-oxoglutarate; this holds also for the ATP activation of the NADPH oxidation reaction.

2. High concentrations of both NADH and NADPH are inhibitory in the reductive amination reaction. 2-Oxoglutarate and glutamate affect the catalytic activity in a different manner depending upon whether the di- or the triphosphopyridine nucleotides are coenzymes of the reaction. NADH inhibition is enhanced at high 2-oxoglutarate concentrations, while NADPH inhibition is diminished. Contrarily to the activating effect of high NAD^+ concentrations, high concentrations of $NADP^+$ inhibit the oxidative deamination reaction.

3. When added to the glutamate deamination assay mixture at 50 mM glutamate, low concentrations of 2-oxoglutarate exhibit competitive inhibition towards glutamate with NAD^+ as coenzyme, and a cooperative type of inhibition with $NADP^+$ as coenzyme of the reaction.

4. It is postulated that the substrates of glutamate dehydrogenase, and especially 2-oxoglutarate, play a regulatory role in the activity of this enzyme.

5. The enzyme is stabilized against inactivation in 10 mM Tris–EDTA (pH 8.0) at 25° by sub-stoichiometric concentrations of ADP and by low concentrations of 2-oxoglutarate, but not by similar or higher concentrations of glutamate. It is concluded that protection must occur through binding of the ligands, and cannot be attributed to ionic effects.

INTRODUCTION

ATP has been reported to be an inhibitor of the NADH oxidation catalyzed by beef-liver glutamate dehydrogenase (L-glutamate:NAD(P) oxidoreductase (deaminating), EC 1.4.1.3), as opposed to the reactions with NAD^+, $NADP^+$ and NADPH which are either activated or unaffected[1]. ATP-inhibition can be abolished by working

at very low NADH concentrations[1,2]. The inhibition caused by high NADH concentrations is known from studies of FRIEDEN[2–5], who has explained it by the existence of a second binding site for NADH on the enzyme in addition to the catalytic site. While it has been repeatedly assumed that binding of the coenzyme changes the properties of the nucleotide-binding site, the influence of the substrate on nucleotide binding has only seldom been taken into consideration[6,7].

The results presented in this paper with both the glutamate deamination and the 2-oxoglutarate amination reactions, using di- as well as triphosphopyridine nucleotides as coenzymes, point to a possible regulatory role of the substrates, and especially of 2-oxoglutarate.

Glutamate dehydrogenase undergoes inactivation in dilute Tris buffer at pH 8.0, and this can be prevented by ADP and several ionic compounds[8]. We have been able to show that the enzyme is stabilized at 25° in the presence of sub-stoichiometric concentrations of ADP and of low levels of 2-oxoglutarate as opposed to glutamate, suggesting that effects other than merely ionic ones are involved.

A brief preliminary report of some of these findings has appeared[9].

EXPERIMENTAL

Crystalline beef-liver glutamate dehydrogenase was obtained from Boehringer and Soehne (Mannheim) as a suspension in 50% glycerol. For the kinetic experiments, dilutions were made in 10 mM sodium phosphate buffer (pH 8.0), containing 0.1 mM EDTA. Under these conditions the enzyme was stable in ice for several hours. The buffer used in the inactivation experiments was 10 mM Tris (pH 8.0), containing 0.1 mM EDTA. NAD^+, $NADP^+$ and NADPH were obtained either from Sigma Chemical Co. or from Boehringer and Soehne. Both Sigma NADH and Boehringer NADPH were prepared fresh daily in dilute Tris buffer (pH 8.0). ATP was obtained from Sigma, while ADP and GDP were purchased from Boehringer. All other reagents were of analytical grade.

The pH of all solutions was adjusted to that of the final reaction mixture (pH 8.0) by the addition of dilute NaOH or HCl, and all solutions were stored at −4°. The standard 3-ml assay mixture contained 10 mM Tris–HCl buffer (pH 8.0). Where used, the concentration of NH_4Cl was 50 mM. The assay mixtures in cuvettes of 1-cm light path were incubated at the temperature of the reaction, 25°. The reaction was initiated by the addition of enzyme. Where different amounts of enzyme were used, the ionic strength of the assay mixtures was properly adjusted. Absorbance changes were recorded with the aid of a Photovolt Corp. recorder Model 43 attached to a Zeiss spectrophotometer PMQ II. An extinction coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$ was used at 340 mμ, and of 3.33 $mM^{-1} \cdot cm^{-1}$ at 366 mμ. With high concentrations of the reduced pyridine nucleotides, absorbance changes were recorded at 366 mμ. This made it possible to work with constant slit opening eliminating artifactual decline in extinction coefficient. Enzyme concentrations were adjusted so as to obtain linear tracings as far as possible, and initial velocities were estimated from the tangent to the earliest part of the curve. Activities were usually expressed as initial rates (*e.g.*, $\Delta A_{340\ m\mu}$/min) per mg protein. The protein concentration of enzyme solutions was estimated spectrophotometrically at 280 mμ using an absorption coefficient of 0.97 for a 1 mg/ml glutamate dehydrogenase solution[10]. In comparing effects obtained in different

experiments, we assumed that the rates calculated per mg protein were independent of enzyme concentration (*cf.* ref. 11).

The inactivation experiments were performed as follows: a 10-fold dilution of the original enzyme solution was prepared in fresh 10 mM Tris buffer (pH 8.0), containing 0.1 mM EDTA, and incubated either unsupplemented (control) or with various additions. Aliquots of 40 μl were removed at various time intervals and assayed in 3-ml mixtures with 0.1 mM NAD^+ as coenzyme and 5 mM sodium glutamate as substrate.

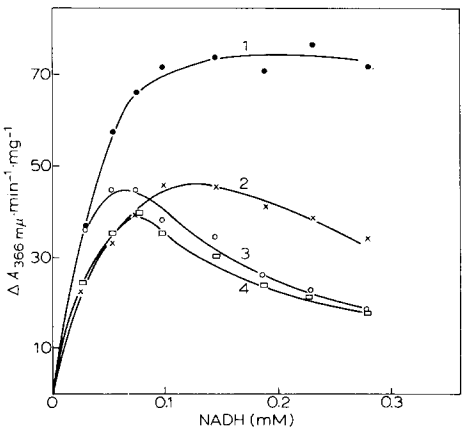

Fig. 1. Effect of ATP on NADH oxidation at varying concentrations of the coenzyme and two constant 2-oxoglutarate concentrations. Curve 1, 1.6 mM 2-oxoglutarate—control; Curve 2, 50 mM 2-oxoglutarate—control; Curve 3, 1.6 mM 2-oxoglutarate *plus* 0.5 mM ATP; Curve 4, 50 mM 2-oxoglutarate *plus* 0.5 mM ATP. The amount of enzyme in the reaction mixture was 1.2 μg in the low 2-oxoglutarate concentration experiment, and 2.5 μg in the 50 mM 2-oxoglutarate concentration experiment. Absorbance changes were recorded at 366 mμ.

RESULTS

Reductive amination of 2-oxoglutarate

The effect of ATP on this reaction was studied at various concentrations of NADH and at two different 2-oxoglutarate concentrations (Fig. 1). It can be seen that Curves 2, 3 and 4 do not obey Michaelis–Menten kinetics. The deviation from the classical shape caused by NADH inhibition is more apparent at 50 mM than at 1.6 mM 2-oxoglutarate. The reaction with 50 mM 2-oxoglutarate is inhibited as compared to the reaction with low 2-oxoglutarate concentration. The inhibition caused by high concentrations of NADH is enhanced by ATP at both low and high 2-oxoglutarate concentrations. However, at 50 mM 2-oxoglutarate ATP acts both as an inhibitor and as an activator of the reaction. In other words, at a concentration of coenzyme (*e.g.*, 25–50 μM) at which the reaction is inhibited by ATP in the presence of low 2-oxoglutarate levels, this purine nucleotide acts as an activator of NADH oxidation at 50 mM 2-oxoglutarate. However small, the activating effect of ATP was observed in 3 different experiments whenever the proper concentrations of coenzyme and substrate were chosen.

In the experiments illustrated in Figs. 2A and B, the coenzyme–substrate–effector relationships were studied in the presence of NADPH as coenzyme of the

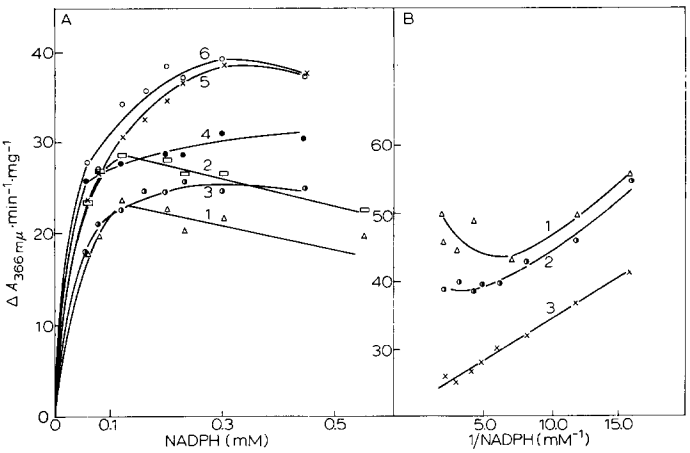

Fig. 2. Effect of ATP on NADPH oxidation at varying concentrations of the coenzyme and 3 constant 2-oxoglutarate concentrations. A. Plot of initial rates *vs.* NADPH concentration. Curve 1, 1.6 mM 2-oxoglutarate—control; Curve 2, 1.6 mM 2-oxoglutarate *plus* 0.1 mM ATP; Curve 3, 2.5 mM 2-oxoglutarate—control; Curve 4, 2.5 mM 2-oxoglutarate *plus* 0.1 mM ATP; Curve 5, 50 mM 2-oxoglutarate—control; Curve 6, 50 mM 2-oxoglutarate *plus* 0.1 mM ATP. The amount of enzyme in the reaction mixture was 2.5 μg. Recording the absorbance changes at 366 mμ made it possible to work at high NADPH levels with constant slit opening. B. Double reciprocal plot of the control lines shown in Fig. 3A. Curve 1, 1.6 mM 2-oxoglutarate; Curve 2, 2.5 mM 2-oxoglutarate; Curve 3, 50 mM 2-oxoglutarate.

reaction. At 50 mM 2-oxoglutarate (Fig. 2B), the double reciprocal plot of initial rate against NADPH concentration is an almost straight line (*cf.* refs. 2, 12). However, high levels of NADPH are inhibitory at low 2-oxoglutarate concentrations, as illustrated by Curves 1 and 2 (Fig. 2B). As in the case of NADH oxidation, NADPH inhibition is related to the concentration of the substrate, 2-oxoglutarate, but in an opposite manner: here, increasing NADPH inhibition is noted at decreasing concentrations of 2-oxoglutarate (compare Curves 5, 3 and 1 in Fig. 2A). While literature data report slight activation or no effect of ATP upon NADPH oxidation, a comparison of Curves 6, 4 and 2 (Fig. 2A) shows that the extent of activation by ATP diminishes as the concentration of 2-oxoglutarate increases (see also Table I).

The results presented in Table I illustrate the above-mentioned decrease of the ATP effect with increasing 2-oxoglutarate concentration (*i.e.*, inhibition in the case of NADH oxidation, and activation in the case of NADPH oxidation) at several levels of coenzyme. The inhibitory effect of high 2-oxoglutarate concentrations is apparent with both coenzymes (compare rates with 25 mM and with 50 mM 2-oxoglutarate).

Oxidative deamination of glutamate

The behaviour of 2-oxoglutarate in the reductive amination reaction suggested that this compound may have an additional effect on the enzyme apart from that of a normal substrate or product. This assumption was tested by adding 2-oxoglutarate to the assay mixture of the glutamate deamination reaction, and as will be shown below, by studying the direct effect of 2-oxoglutarate upon the enzyme in a series of inactivation experiments.

Normally one would expect 2-oxoglutarate, a product of glutamate deamination,

TABLE I

RELATIONSHIPS BETWEEN REDUCED COENZYME–2-OXOGLUTARATE CONCENTRATIONS AND THE EFFECT OF ATP UPON THE REACTION

Figures in the table represent initial rates per mg protein (*c*, control experiment; *ATP*, with ATP). For NADH oxidation, the concentration of ATP was 0.1 mM and the amount of enzyme in the reaction mixture was 1.2 μg. Absorbance changes were recorded at 340 mμ. For NADPH oxidation, the concentration of ATP was 0.5 mM, and the amount of enzyme in the reaction mixture was 2.4 μg. Absorbance changes were recorded at 366 mμ and recalculated for values at 340 mμ.

2-Oxoglutarate (mM)	*1.6*		*5.0*		*10.0*		*25.0*		*50.0*	
	c	*ATP*	*c*	*ATP*	*c*	*ATP*	*c*	*ATP*	*c*	*ATP*
NADH (μM)										
85	—	—	—	—	104	77	105	92	96	89
% effect*						−26		−12		−7
100	—	—	99	54	104	69	108	88	100	88
% effect				−45		−34		−19		−12
120	—	—	—	—	110	72	110	86	90	83
% effect						−35		−22		−8
150		—	100	45	—	—	—	—	80	69
% effect				−55						−14
NADPH (μM)										
85	22	33		—	39	50	38	42	30	34
% effect		+33				+22		+10		+11
330**	26	35		—	47	58	49	61	42	49
% effect		+26				+19		+19		+14

* Negative signs indicate inhibition, positive ones, activation.

** Note that the high NADPH concentration used in this experiment is still below the inhibitory NADPH concentration (see Fig. 2).

to exhibit product inhibition. However, the results illustrated in Fig. 3 show that in the low 2-oxoglutarate concentration range (see insert) the reaction of $NADP^+$ reduction, unlike that of NAD^+ reduction, is slightly activated. This small degree of activation was observed in 3 experiments, with 2 different batches of enzyme. When the substrate (glutamate) is reduced to 10 mM instead of the 50 mM used in the experiment illustrated in Fig. 3, the initial rise in $NADP^+$ reduction rate is no longer observed. The decline in activity under the influence of increasing 2-oxoglutarate concentrations tends to level off.

The nature of the inhibition exhibited by 2-oxoglutarate in the glutamate deamination reaction was studied in the experiments presented in Figs. 4A and 4B. At the low concentration tested, 2-oxoglutarate appears to be a competitive inhibitor of glutamate in the NAD^+ reduction as opposed to the cooperative type of inhibition observed in the case of $NADP^+$ reduction. At the high concentration of 2-oxoglutarate, a type of inhibition which may be defined as partial non-competitive was observed with both coenzymes. The non-linearity of the plots obtained from the experiments with 20 mM 2-oxoglutarate may be open to discussion, but since a similar distribution of experimental points recurred in 2 or 3 different experiments, the possibility of cooperative effects has to be envisaged.

The cooperative type of inhibition shown by 2-oxoglutarate in the glutamate deamination reaction with $NADP^+$ as coenzyme was found also with glutamate as

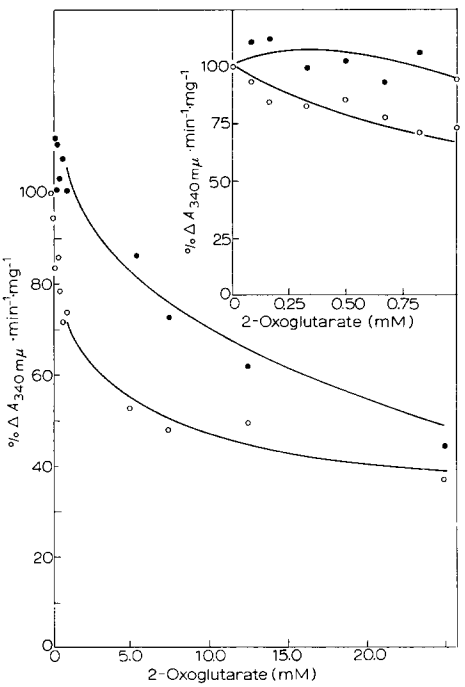

Fig. 3. Effect of 2-oxoglutarate on the reaction of glutamate oxidation at constant concentrations of NAD^+ and $NADP^+$. The upper curve describes the course of the $NADP^+$ to NADPH reaction at 0.1 mM $NADP^+$ and 50 mM glutamate; lower curve, NAD^+ to NADH reaction at 0.1 mM NAD^+ and 50 mM glutamate. The amount of enzyme in the reaction mixture was 24 μg for the NAD^+ ,and 38 μg for the $NADP^+$ reaction. Absorbance changes were read at 340 mμ. The insert represents the same experiments at an enlarged scale of the 0–1 mM 2-oxoglutarate concentration range.

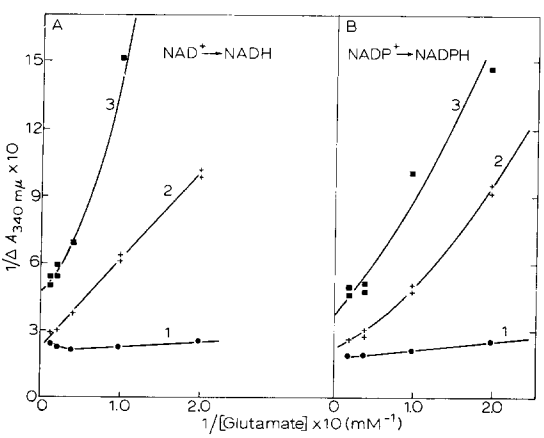

Fig. 4. Effect of low and high 2-oxoglutarate concentration on the reaction of glutamate oxidation. A. Double reciprocal plot of initial rate *vs.* glutamate concentration with NAD^+ (0.1 mM) as coenzyme. Curve 1, control; Curve 2, with 3 mM 2-oxoglutarate; Curve 3, with 20 mM 2-oxoglutarate. The amount of enzyme in the reaction mixture was 48 μg. B. Double reciprocal plot of initial rate against glutamate concentration with $NADP^+$ (0.1 mM) as coenzyme. Curve 1, control; Curve 2, with 3 mM 2-oxoglutarate; Curve 3, with 20 mM 2-oxoglutarate. The amount of enzyme in the reaction mixture was 100 μg.

inhibitor of the 2-oxoglutarate amination with NADPH. Also in this case, slight activation of NADPH as opposed to NADH oxidation was obtained when the substrate concentration was 50 mM. At 10 mM 2-oxoglutarate, increasing glutamate levels caused inhibition with both coenzymes. Plotting $1/v$ *vs.* glutamate concentration by the method of DIXON[13] at 2 concentrations of 2-oxoglutarate, we found that the 2 lines (representing 10 and 50 mM 2-oxoglutarate) crossed in the first quadrant. This can be explained by the existence of 2 different forms of the enzyme at low and at high 2-oxoglutarate concentrations.

These experiments were performed with 0.1 mM coenzyme. As NAD^+ is known to exhibit a dualistic effect[3,14–18] we decided to vary NAD^+ resp. $NADP^+$ concentrations and work at a constant concentration of 2-oxoglutarate. Unlike other inhibitors of the glutamate dehydrogenase reaction which exhibited noncompetitive inhibition with regard to NAD^+ at low levels of the coenzyme[17], 2-oxoglutarate showed noncompetitive inhibition in the high concentration range (see Fig. 5A). The effect of 2-oxoglutarate upon $NADP^+$ reduction at varying concentrations of the coenzyme is shown in Fig. 5B. Contrarily to previous reports, in which linear Lineweaver–Burk plots were obtained in experiments with $NADP^+$ (see refs. 14–16), the results illustrated in Fig. 5B point to inhibition at high coenzyme concentrations. However, a straight line was obtained in the presence of 1 mM 2-oxoglutarate, which as seen in Fig. 3, has no inhibitory effect upon the reduction of 0.1 mM $NADP^+$.

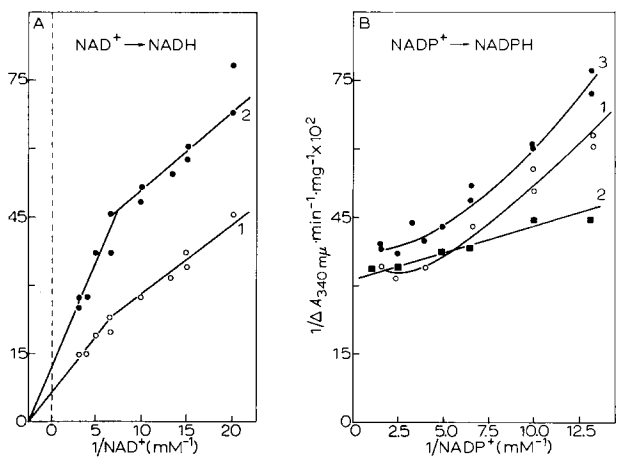

Fig. 5. Effect of 2-oxoglutarate on glutamate oxidation at various concentrations of coenzyme. A. Double reciprocal plot of initial rate *vs.* NAD^+ concentration at 50 mM glutamate. Curve 1, control; Curve 2, with 3.75 mM 2-oxoglutarate. The amount of enzyme in the reaction mixture varied between 10 and 50 μg. B. Double reciprocal plot of initial rate *vs.* $NADP^+$ concentration at a glutamate concentration of 50 mM. Curve 1, control; Curve 2, with 1 mM 2-oxoglutarate; Curve 3, with 5 mM 2-oxoglutarate. The amount of enzyme in the reaction mixture varied between 25 and 70 μg. Absorbance changes were read at 340 mμ.

In order to find out whether variation of the substrate concentration affects the coenzyme binding site in the oxidative deamination reaction, we investigated the influence of glutamate concentration with NAD^+ as coenzyme and noted that the biphasicity of the line (due to NAD^+ activation) diminished as the concentration of glutamate was increased from 5 through 10 to 25 mM.

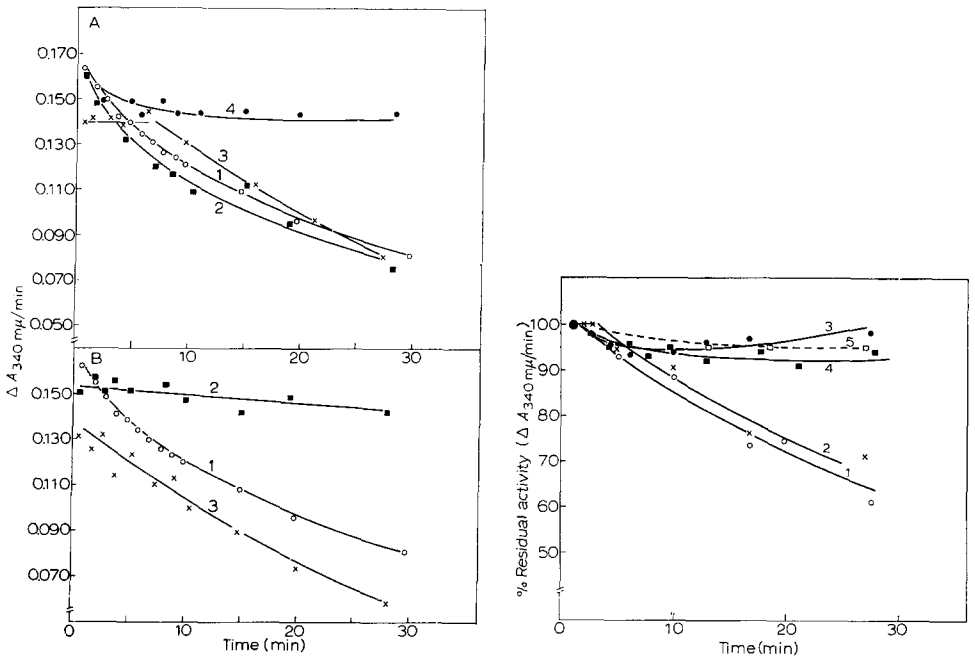

Fig. 6. Effect of oxidized and reduced coenzymes upon the stability of glutamate dehydrogenase. A. Initial rates *vs.* time. Curve 1, control; Curve 2, with 0.1 mM NAD^+; Curve 3, with 0.1 mM $NADP^+$. Curve 4, with a mixture of 1 mM 2-oxoglutarate and 0.1 mM $NADP^+$. B. Initial rates *vs.* time. Curve 1, control; Curve 2, with 0.1 mM NADH; Curve 3, with 0.2 mM NADPH. In the control experiment, the unsupplemented enzyme (1 mg/ml) was allowed to inactivate in 10 mM Tris (pH 8.0), at 25°. Equal aliquots (40 μg) were removed at the indicated time intervals and assayed at 340 mμ with 5 mM glutamate and 0.1 mM NAD^+.

Fig. 7. Effect of various ligands upon the stability of glutamate dehydrogenase (per cent residual activity *vs.* time). Curve 1, control; Curve 2, with 2.5 mM glutamate; Curve 3, with 0.1 mM 2-oxoglutarate; Curve 4, with 10 μM ADP; Curve 5, with 0.1 mM ADP. Other conditions as in legend to Fig. 6.

Inactivation experiments

As illustrated in Fig. 6, a decline occurred in the activity of the enzyme when incubated in 10 mM Tris buffer–EDTA (pH 8.0), at 25°. The presence of NAD^+ and NADPH (Curves 2 and 3 in Figs. 6A and B, respectively) did not appear to affect this decline to any significant extent. Stabilization was obtained in the presence of NADH, while $NADP^+$ induced a lag period of about 7 min in the inactivation process. A drop in initial rate was invariably observed in the first minute after addition of $NADP^+$ and NADPH.

The effect of 2-oxoglutarate upon the enzyme interested us in view of the above-described kinetic results. Its effect upon enzyme stability was compared to that of glutamate and ADP. The results of these experiments are presented in Fig. 7. As seen in this figure, stabilization was achieved at a concentration of 2-oxoglutarate as low as 0.1 mM, while 2.5 mM glutamate could hardly prevent inactivation (stabilization in the presence of glutamate was found at 5 mM). ADP protected the enzyme against inactivation not only at 0.1 mM (Curve 5), but also at a concentration of 10 μM which

was sub-stoichiometric at the enzyme concentration of this experiment (see DISCUSSION). It should be noted that the precise extent of inactivation of the enzyme (control) was subject to some variation (slight changes in pH have a significant effect) but the stabilizing effect of 10 μM ADP was invariably found.

At a concentration of 0.1 mM or lower, GDP had no effect upon inactivation of the enzyme under these conditions.

A slight turbidity developed during inactivation which could be followed by an increase in the ultraviolet absorption of the enzyme. Those compounds which counteracted the decline in catalytic activity also prevented the increase in absorption. They did not induce any shifts in the normal spectrum of the enzyme.

DISCUSSION

The data presented in this paper confirm the assumption that several forms of glutamate dehydrogenase are in equilibrium in solution. The direction of the equilibrium is determined in each case not only by the choice of the coenzyme and of the purine nucleotide effector, but also by the concentrations of the substrate or product of the reaction.

At high concentrations (*e.g.*, 50 mM) of 2-oxoglutarate, both NADH and NADPH oxidations proceed at a lower rate than, for instance, at 25 mM 2-oxoglutarate. This inhibition is probably the result of a combined substrate–coenzyme effect, as it is dependent on coenzyme concentration. The inhibition caused by high concentrations of the reduced coenzymes is enhanced in the case of NADH, and diminished in the case of NADPH—at high 2-oxoglutarate concentrations. With increasing concentrations of 2-oxoglutarate, there is a decrease of ATP inhibition in the NADH reaction, and of ATP activation in the NADPH reaction. The effect of 2-oxoglutarate upon glutamate deamination differs depending upon the concentrations of glutamate and depending upon which of the 2 nucleotides is utilized as cofactor. At 50 mM glutamate and 0.1 mM pyridine nucleotide, low levels of 2-oxoglutarate slightly activate $NADP^+$ reduction and inhibit NAD^+ reduction. Kinetic plots indicate that 2-oxoglutarate is not merely a competitive inhibitor of glutamate, and suggest that cooperative effects are involved.

The interplay between coenzyme and effector concentrations is mentioned in the literature. FRIEDEN[1,2] showed that ATP-inhibition of 2-oxoglutarate amination with NADH could be abolished by working at low concentrations of this coenzyme. Separate catalytic and regulator sites were postulated[2] for NADH and NAD^+, and only one catalytic site for NADPH and $NADP^+$. In a recent work[19] it has been shown that NADH binding is not related to the concentration-dependent aggregation of the enzyme, and shows a simple dependence on NADH concentration. On the basis of their results, the authors assume that there is no regulator site for NADH. Our finding of the inhibitory action of both NADPH and NADP (see also ref. 20 for Nitrosomonas glutamate dehydrogenase) also casts doubt on the hypothesis of separate pyridine nucleotide binding sites. In this connection, it is interesting to mention the work of BEN-HAYYIM, HOCHMAN AND AVRON[21] who on the basis of the inhibition of NAD^+ reduction by 2′-phosphoadenosine diphosphate ribose—a specific inhibitor of $NADP^+$ enzymes—conclude that glutamate dehydrogenase, which may use either NAD^+ or $NADP^+$

as cofactors, must have no NAD-specific binding site but rather a non-specific common binding site for both nucleotides.

The mutual relationships between glutamate and NADH (or NADPH) concentrations, which appear from optical rotatory dispersion studies performed with pork liver glutamate dehydrogenase[7] lead to the conclusion that the enzyme exists in 2 allosteric states, whereby fixation of the ligand NADH in the presence of glutamate causes transition of one state into the other. SUND[22] obtained parallel lines for the pH-dependence curves of v_{max} and K_{max} at low and high NAD^+ concentrations, showing that binding of active and activating concentrations of NAD^+ takes place through the same functional groups on the enzyme. He concluded that activation is due to a conformational change of the enzyme under the influence of NAD^+. Similarly one may assume, for example, that a less active conformation of the enzyme induced by high NADH concentrations in the presence of 2-oxoglutarate is promoted by ATP and GTP, but counteracted by ADP.

Recent work performed on D-amino acid oxidase[23] shows that the observed substrate inhibition is due to conformations with different activities having different affinities for the substrates and effectors. Activity cannot be related to the ligand saturation function[24]. Only the state functions can be related to activity (*cf.* ref. 24) while keeping in mind that in two-substrate reactions, v_{max} and K_{max} are dependent upon the concentration of both substrates. As different reagents may have various affinities to conformations of different activities, one substrate can act as an allosteric effector and affect as such the kinetic parameters of the second substrate. The result will be different according to the kinetic mechanism operating, *i.e.*, modified enzyme mechanism, ordered mechanism or random mechanism (see ref. 23).

The regulatory function of the pyridine nucleotides (coenzymes) and of the purine nucleotides (allosteric effectors) has often been mentioned in discussing the role of glutamate dehydrogenase in metabolic control. In our opinion, the possible regulatory function of substrates such as 2-oxoglutarate has been unjustly neglected in studies with the pure enzyme. The experiments described above demonstrate that markedly different results are obtained when the concentration of either 2-oxoglutarate or glutamate is varied. This is consistent with the idea that conformations with different affinities for the two substrates play a role in the catalytic mechanism. In this connection it is interesting to mention the work of PAPA *et al.*[28], who stressed the importance of 2-oxoglutarate concentration in the function of the enzyme in isolated mitochondrial particles and found stimulation of glutamate oxidation by removal of 2-oxoglutarate. Very little 2-oxoglutarate accumulates during glutamate oxidation in the presence of malonate, which was shown to increase the permeability of the mitochondrial membrane for 2-oxoglutarate[27]. Glutamate deamination is increased in the presence of malonate, while there is only a partial reduction of $NADP^+$; the factor controlling the activity of the enzyme in mitochondria is the oxidoreduction state of NADP[28].

The above-mentioned effects obtained with 2-oxoglutarate and glutamate were not found with the second substrate of the reverse reaction, the ammonium ion. Apparently normal Michaelis–Menten curves were obtained with NH_4Cl up to concentrations of 100 mM, and the known effects of ATP and GTP upon either NADH or NADPH oxidations were not affected by NH_4Cl concentration.

Our results obtained in the inactivation experiments provide additional infor-

mation with regard to the effect of ligands upon enzyme conformation. These experiments were performed at an enzyme concentration of 1 mg/ml, which is about 20 μM if we take 50 000 as the approximate molecular weight of the smallest binding unit (*cf.* refs. 19, 25, 26). The effect of the coenzymes and other ligands upon enzyme stability was distinguished from their effect upon catalytic activity. Results of separate experiments which were not illustrated here proved that neither activation nor inhibition of the catalytic reaction was caused by the effectors, which underwent a 75-fold dilution in the assay mixture. Although unexplained so far, the stabilizing effect of a concentration of 2-oxoglutarate as low as 0.1 mM seems remarkable, the more so because glutamate at a concentration 25 times higher did not succeed in achieving a similar result. This difference between 2-oxoglutarate and glutamate indicates clearly that in this case stabilization cannot be explained by the protective effect of ionic compounds (contrast DI PRISCO AND STRECKER[8]), but must be the result of binding of the ligands to the enzyme. This is in contradiction with the previously postulated lack of binding of 2-oxoglutarate to glutamate dehydrogenase in the absence of the coenzyme[29].

Assuming that 10 μM ADP is fully bound by the enzyme, the fact that more than 50% stabilization is obtained at this concentration suggests that the shuttling of ADP between the enzyme molecules is sufficiently rapid to prevent unfolding and subsequent inactivation of the enzyme. This means also that the enzyme must spend a sufficient length of time in the free form before unfolding can occur.

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