BBA 65916

RELATIONSHIP BETWEEN STABILITY AND STATE OF ASSOCIATION IN GLUTAMATE DEHYDROGENASE

B. EISENKRAFT, J. B. VAN DORT AND C. VEEGER
Department of Biochemistry, Agricultural University, Wageningen (The Netherlands)

(Received January 3rd, 1969)
(Revised manuscript received April 4th, 1969)

SUMMARY

1. Inactivation of glutamate dehydrogenase (L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3) in 10 mM Tris–HCl 0.1 mM EDTA (pH 8.0) at 25° was stimulated by the reduced diphosphopyridine nucleotides at concentrations as low as 5 and 10 μM, but counteracted at coenzyme concentrations above 100–200 μM. The inactivation promoting effect of 10 μM NADPH and of 0.5 M urea was fully counteracted by ADP and partially prevented by 2-oxoglutarate.

2. Addition of ADP to a partially inactivated enzyme 10 min after dilution in 10 mM Tris buffer (pH 8.0) at 25° resulted in restoration of the original activity. Addition of ADP at later stages led to partial reactivation.

3. Arrhenius plots constructed by means of the rate constants of inactivation calculated for various temperatures at an enzyme concentration of 0.1 mg/ml yielded energies of activation of about 20 kcal/mole. NADPH at a concentration of 10 μM causes an increase in ΔS^* of 1.4 e.u. at the same E_A.

4. A linear relationship exists between the rate constants of inactivation at various concentrations of enzyme and the relative amounts of monomer present. The limiting step in the process of inactivation appears to be formation of a catalytically inactive monomer, probably followed by its dissociation into smaller inactive subunits.

INTRODUCTION

It has been pointed out earlier[1–4,21] that apart from their effect on reaction rates, substrates and other ligands affect the stability of glutamate dehydrogenase (L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3). It has also been claimed that the conformational changes which result from this type of interaction play a role in the general regulation of enzyme activity[5,6].

TANFORD[7] defines "protein denaturation" as a major change from the original native structure without alteration of the amino acid sequence. We have made use of the property of enzyme instability in dilute Tris–HCl buffer (pH 8.0) and have tried to

influence the behavior of the enzyme by adding various compounds to the incubation system.

In a previous article[8] we pointed out that the protection offered by compounds such as ADP and 2-oxoglutarate against inactivation in 10 mM Tris–HCl buffer (pH 8.0) at 25° cannot be attributed to ionic effects. The findings presented in this paper corroborate and extend our previous observations. Investigation of inactivation, a process following pseudo first-order kinetics, at various enzyme concentrations has led to the finding of a straight line relationship between the rate constants of inactivation and the relative amounts of monomer of about 310 000 molecular weight at the corresponding enzyme concentrations. The limiting step in the process of inactivation appears to be the formation of a catalytically inactive monomer, which is followed by fragmentation into smaller units.

MATERIALS AND METHODS

Crystalline beef liver glutamate dehydrogenase in 50% glycerol was purchased from Boehringer (Mannheim). NAD^+ and $NADP^+$ were obtained from Sigma Chemical Co. and were kept frozen at pH 7. NADH (Sigma) and NADPH (Boehringer) were prepared fresh daily in dilute Tris–HCl buffer (pH 8.0). ATP was obtained from Sigma, while ADP, GDP and GTP were from Boehringer, as were most other compounds.

Incubation of the enzyme for the purpose of studying inactivation was performed in 10 mM of Tris–HCl buffer (pH 8.0) with 0.1 mM EDTA at 25°, except when the effect of temperature was studied. In a few cases, as mentioned under the individual experiments, 10 mM of phosphate buffer with 0.1 mM of EDTA (pH 8.0) was used. The dilute Tris–HCl buffer was prepared fresh daily from a 1 M stock solution which was made once a week or once in 2 weeks and was kept in the refrigerator. The pH of the 10 mM solution was adjusted with an accuracy of $\pm$ 0.01 pH units by means of a Radiometer Titrator 11 (Copenhagen) at the incubation temperature. Whenever incubation was performed in the presence of some compound, the enzyme was diluted in buffer solution which contained that compound.

Equal aliquots were removed at given times and were added to 3-ml assay mixtures at 25°, containing 0.1 mM NAD^+ and 5 mM sodium glutamate in 10 mM Tris–HCl (pH 8.0). In working with various dilutions of enzyme, care was taken to add a constant amount of protein to the assay mixtures. In order to eliminate possible effects due to the presence of various amounts of glycerol, we tested inactivation of a constant (low) concentration of enzyme at various glycerol concentrations and found no significant difference in the inactivation rate over the glycerol concentration range of 0.25–2.0%.

Absorbance changes at 340 nm were recorded in 1-cm light path cuvettes with the aid of a Photovolt Corp. recorder Model 43 attached to a Zeiss spectrophotometer PMQ II. The protein concentration of enzyme solutions was estimated spectrophotometrically at 280 nm using an absorption coefficient of 0.97 for a 1 mg/ml glutamate dehydrogenase solution[9].

RESULTS AND DISCUSSION

In the following experiments, catalytic activity was the main criterion for

characterizing the state of stability of the enzyme incubated in dilute Tris–HCl buffer (pH 8.0). The secondary effects which the compounds added to the incubation mixture and which the enzyme dilution factor might have in the assay were eliminated by performing suitable control experiments. In such a manner, whatever conclusions are being drawn about the state of stability of the enzyme, these refer to the conditions of incubation prior to the catalytic assay. A large number of the experiments described below were performed at an enzyme concentration of 1 mg/ml. This gave at 25° a convenient decline in activity within 30 min for a reliable study of both protective and inactivation promoting agents in 10 mM Tris–HCl buffer (pH 8.0).

The effects of NADH and NADPH upon glutamate dehydrogenase stability are greatly dependent upon their respective concentrations. This is illustrated for NADH in Fig. 1. It is remarkable that concentrations as low as 5 and 10 μM are most effective in enhancing the inactivation of 20 μM enzyme (based on a chain molecular weight of about 50 000). The smallest binding unit is taken into consideration, as it is assumed that there are 4–6 binding sites for the reduced coenzymes per catalytic unit of 300 000 mol. wt. (refs. 10, 11). Practically no inactivation occurred in the presence of 100 μM NADH. NADPH at the same concentration had no significant effect on the rate of inactivation of the enzyme.

We reported earlier[8] that ADP can protect the enzyme (20 μM) against inactivation under our conditions, at a concentration of 10 μM. When the enzyme was incubated with 100 μM ADP or with a mixture of ADP and one of the inactivation

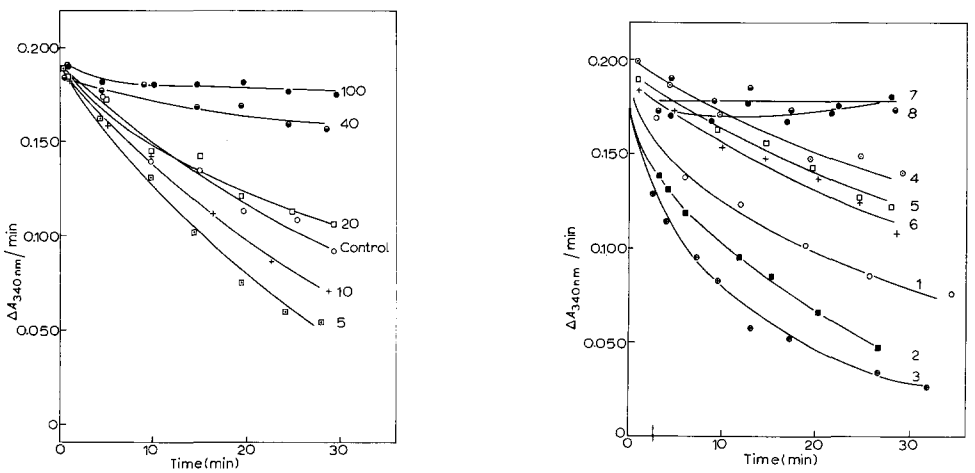


Fig. 1. Effect of NADH upon enzyme stability. The enzyme (1 mg/ml) was incubated in 10 mM Tris–HCl buffer, 0.1 mM EDTA (pH 8.0) at 25° with NADH at the concentrations given in the figure (in μM). Aliquots of 0.04 ml were added at the indicated times to the assay mixture which contained 5 mM sodium glutamate and 0.1 mM NAD^+ in 10 mM Tris–HCl buffer, 0.1 mM EDTA (pH 8.0), in a final volume of 3 ml. Initial rates were measured at 25°.

Fig. 2. Effect of various ligands and of combinations of ligands upon enzyme stability. Curve 1, control experiment; Curve 2, enzyme incubated with 0.5 M urea; Curve 3, enzyme incubated with 10 μM NADPH; Curve 4, enzyme *plus* 100 μM 2-oxoglutarate; Curve 5, enzyme *plus* 0.5 M urea and 100 μM 2-oxoglutarate; Curve 6, enzyme *plus* 10 μM NADPH and 100 μM 2-oxoglutarate; Curve 7, enzyme *plus* 10 μM NADPH and 100 μM ADP; Curve 8, enzyme *plus* 0.5 M urea and 100 μM ADP. The protein concentration was 1 mg/ml. Assay conditions as in legend to Fig. 1.

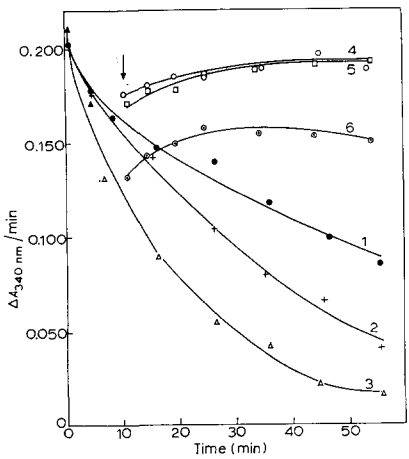
promoting compounds (10 μM NADPH or 0.5 M urea), very little decrease in catalytic activity was found, in contrast to enzymes incubated in the presence of urea or NADPH alone (Fig. 2).

The substrate 2-oxoglutarate protects against inactivation although less efficiently than ADP. In previous experiments 2-oxoglutarate proved to be a better protective agent than glutamate, as it maintained the enzyme at a higher level of activity at concentrations of 2-oxoglutarate several orders of magnitude below those usually used in the catalytic assay. As a matter of fact, full stabilization by 0.1 mM 2-oxoglutarate was reported[8]. We have not been able to reproduce this effect, and a possible reason for this is that the rate of inactivation is dependent among other factors on the age of the enzyme batch, as noted earlier by FRIEDEN[2]. In the experiment illustrated in Fig. 2, 2-oxoglutarate counteracts, at least in part, the enhanced inactivation induced by urea and NADPH. Quantitatively, the reproducibility of the results in experiments with 2-oxoglutarate was not very satisfactory. The recurring scatter of the experimental points suggests that several intermediates with various catalytic activities are in equilibrium. It is noteworthy that 2-oxoglutarate exerts its protective effect also, and even better, at concentrations at which it causes inhibition in the assay. Obviously, its effect on stability is not simply related to its role as substrate in the catalytic reaction.

The problem of the reversibility of inactivation could not be readily investigated by methods such as dialysis or gel filtration due to instability of the enzyme in dilute Tris–HCl buffer. We made use of a partially inactivated enzyme obtained after brief incubation and tried to reactivate it by means of stabilizing agents. Usually about 20–25% of the initial activity was lost by the 10th min in the case of 1 mg/ml enzyme. Under these conditions, addition of 100 μM ADP led to almost full recovery of the initial activity at the end of about 60 min (Fig. 3). When the enzyme was inactivated in the presence of 10 μM NADPH, addition of ADP after 10 min induced only partial regain of activity. At first we attributed this result to the greater extent of inactivation of the enzyme after a 10-min incubation with NADPH. In another experiment ADP was added after 5 min, corresponding to the level of activity reached by the control after 10 min. The results showed that ADP was probably less efficient in reactivating the NADPH inactivated enzyme, suggesting that different forms of the enzyme appear according to whether inactivation takes place in the presence of NADPH or of urea.

Some reactivation did take place when ADP was added to the enzyme at a more advanced stage of inactivation (about 35–40%).

In an attempt to calculate activation energies characteristic of the inactivation process, we plotted the rate constants calculated at various temperatures against the reciprocal of the absolute temperature. The plots were linear in the 8–25° range. Below 8° large inaccuracy was introduced by the low inactivation rates. Energies of activation calculated from the slopes of the lines in several experiments were in the range of 20 kcal/mole for 0.1 mg/ml enzyme. A similar value was obtained in experiments in which 10 μM NADPH were added to the enzyme undergoing inactivation (Fig. 4, line 2). However, inactivation proceeded at a higher rate in the latter case, resulting in an increase of 1.4 e.u. in ΔS^* in the presence of NADPH. The ΔH of chymotrypsinogen inactivation (pH 2.0–3.0) varies between 99.6 and 143 kcal/mole (ref. 12). Obviously, the changes occurring in glutamate dehydrogenase under the above conditions of inactivation are of a different nature.



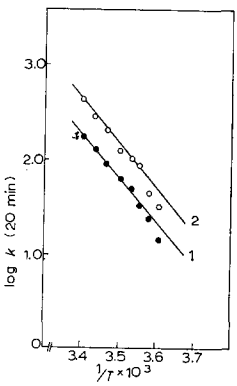


Fig. 3. Addition of ADP to a partially inactivated enzyme. Curve 1, control experiment; Curve 2, enzyme incubated with 0.5 M urea; Curve 3, enzyme incubated with 10 μM NADPH; Curve 4, 100 μM ADP added to the control; Curve 5, 100 μM ADP added to enzyme inactivated in the presence of 0.5 M urea; Curve 6, 100 μM ADP added to enzyme inactivated in the presence of 10 μM NADPH. The arrow represents the moment of ADP addition. Protein concentration, 0.8 mg/ml. Assay conditions as in legend to Fig. 1.

Fig. 4. Arrhenius plots of enzyme inactivation. Line 1, control enzyme; Line 2, enzyme incubated with 10 μM NADPH. The points represent averages from three independent experiments. Protein concentration, 0.1 mg/ml. Assay conditions as in legend to Fig. 1. The activation energy calculated from the slopes of the lines is about 20 kcal/mole. Pseudo first-order rate constants of inactivation were obtained as described in legend to Fig. 5, except that the values of k refer to periods of 20 min.

In view of the concentration-dependent dissociation of glutamate dehydrogenase, we were interested in the relationship between the rate of inactivation and enzyme concentration. As inactivation followed pseudo first-order kinetics, rate constants could be calculated from the lines in Fig. 5. As seen in the insert, the inactivation rate reaches a limiting value at high dilutions. At enzyme concentrations of 0.025–0.05 mg/ml, the molecular weight of the enzyme approaches the molecular weight of the monomer, which is the smallest catalytic subunit[13,14]. This suggested that there may be a relationship between the rate of inactivation and the fraction of monomer present at a given enzyme concentration. In order to test this idea, we made use of the method of STEINER[15] which makes it possible to calculate the consecutive association constants and the types of polymer species present in a system from the concentration dependence of molecular weight. The results of recent light scattering and sedimentation experiments[13,14] suggest a stepwise association–dissociation equilibrium for glutamate dehydrogenase. In the limit $c = 0$, the molecular weight of the enzymically active oligomer is about 310 000. The average value of the molecular weight appears to increase without limit to over 2 000 000 at 8 mg/ml (ref. 13).

When a monomer A_1 associates to form a series of polymers, A_2 (dimer), A_3 (trimer), *etc.*, and if we assume that the system attains chemical equilibrium very rapidly, the concentrations in mg/ml of A_1 and A_2, denoted by c_1 and c_2, are related by the equation $c_2 = K_2 c_1^2$, where K_2 is the equilibrium constant for the formation of A_2 from A_1 (ref. 16). Similarly, $c_3 = K_2 K_3 c_1^3$, where c_3 is the concentration of the tiimer,

and K_3 is the equilibrium constant for the reaction $A_1 + A_2 \rightleftharpoons A_3$. The total concentration $c = c_1 + c_2 + \ldots + c_i$ can be expressed as a function of c_1 only.

The weight-average molecular weight M_w of A_1, A_2, . . A_i is given by

$$M_w = \sum^{i} c_i M_i / \sum^{i} c_i \tag{1}$$

where c_i and M_i are the concentration and molecular weight, respectively, of species i. Substituting in Eqn. 1 the above relations for c_2, c_3, *etc.*, one obtains

$$M_w c / M_1 c_1 = 1 + 2K_2 c_1 + 3K_2 K_3 c_1^2 + 4K_2 K_3 K_4 c_1^3, \text{ etc.} \tag{2}$$

In this way, the problem of calculating the equilibrium constants K_2 and K_3 is reduced to measuring $M_w(c)$. The weight fraction of the monomer (c_1/c) can be evaluated using the formula derived by STEINER[15]:

$$\ln c_1/c = \int_0^c (M_1/M_w - 1)/c \, dc \tag{3}$$

Graphical integration of $(M_1/M_w - 1)/c$ makes it possible to obtain c_1/c, and thus c_1, the concentration of the monomer at a given total concentration c. In our calculations we used the $M_w(c)$ data obtained by EISENBERG AND TOMKINS[13] from light scattering experiments at 25° which yielded an average molecular weight of 310 000 for the monomer (see also ref. 14). These data enabled us to calculate the molecular species composition at various enzyme concentrations up to the trimer (Table I) by means of

TABLE I

CHANGE IN MOLECULAR SPECIES COMPOSITION WITH ENZYME CONCENTRATION

Calculations based on data from refs. 13 and 15.

Total concn. (mg/ml)	*Monomer*		*Dimer*		*Trimer*	
	mg/ml	*%*	*mg/ml*	*%*	*mg/ml*	*%*
1.0	0.25	25	0.31	31	0.127	12.7
0.4	0.17	42	0.14	35	0.038	9.5
0.2	0.11	55	0.066	33	0.013	6.5
0.1	0.07	70	0.026	26	—	—
0.05	0.04	80	0.009	17	—	—
0.025	0.02	88	0.003	11	—	—

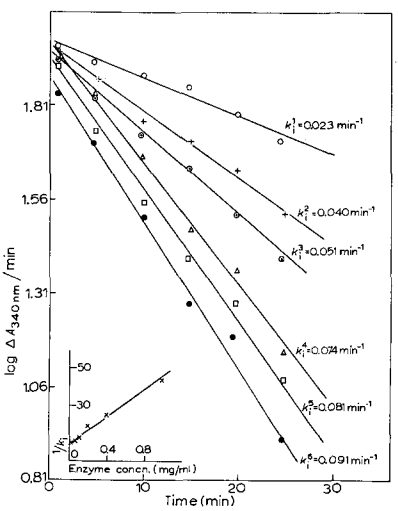
equilibrium constants obtained at lim $c \to 0$ from the slopes of the plots of $M_w c/M_1 c_1$ *vs.* c_1, and $M_w c/M_1 c_1 - 2K_2 c_1$ *vs.* c_1^2, as shown by Eqn. 2. Both K_2 and K_3 can also be obtained from the intercept (at $c_1 = 0$) and the slope of a plot for $[(M_w c/M_1 c_1) - 1]/c_1$ *vs.* c_1 if they are independent of c (ref. 16). The values of the association constants obtained by both methods amount to $7.8 \cdot 10^5$ l·mole^{-1} for K_2', and $3.5 \cdot 10^5$ l·mole^{-1} for K_3', where $K_2' = K_2 M_1/2$, and $K_3' = 2K_3 M_1/3$.

In principle it is possible to determine all equilibrium constants up to K_i, but in practice it is difficult to determine constants higher than K_3 because of the limited accuracy obtained in the measurement of $M_w(c)$, and because the error introduced in the determination of c_1 is accumulated in the subsequent calculation steps. Due to this difficulty, we regard the value of K_3 and the trimer concentrations presented in Table I

as approximate. We are aware of the existence of polymers higher than the trimer above 0.1 mg/ml enzyme because of the upward curvature found in the plot of $[(M_w c/M_1 c_1) - 1]/c_1$ *vs.* c_1, as would be expected in such a case (*cf.* ref. 16) according to Eqn. 2.

When the rate constants of inactivation obtained from the lines in Fig. 5 are plotted against the relative amounts of mono-, di- and trimer at the corresponding total concentrations, a straight line relationship is obtained for the monomer variation, thus confirming our original assumption that the monomer is the active species in the inactivation process (see Fig. 6). One may argue whether it is correct to use molecular weight determinations[13] made in 0.2 M phosphate buffer (pH 7) in calculations referring to enzyme diluted in 10 mM Tris–HCl buffer (pH 8.0). As a matter of fact, such determinations cannot be made on an instable enzyme. In taking the molecular weight distribution determined in phosphate buffer as a basis for our calculations, we have assumed that it holds at least for the state of the enzyme at time zero (*i.e.*, just prior to inactivation) under our conditions. In this respect, we should like to quote the data of FRIEDEN[17] who found no significant differences in the sedimentation pattern of the enzyme between phosphate and Tris–acetate buffers, as well as an essentially constant sedimentation coefficient from pH 6–10 in the latter buffer.

The linear pattern of monomer variation was reproducible despite variations in the absolute value of the inactivation constants. In this respect, it should be noted that our values are of the same order of magnitude as those reported earlier[2] for NADPH inactivation of the enzyme. As seen in Fig. 6, the monomer line extrapolates



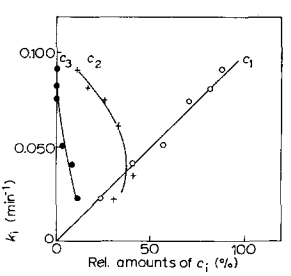


Fig. 5. Dependence of enzyme inactivation upon protein concentration. Initial rates at various time intervals after inactivation started were recalculated as percent of the extrapolated catalytic activity at zero time, and the logarithms of these values were plotted against time. Assay conditions were as described in legend to Fig. 1. The rate constants of inactivation are given in the figure. Protein concentrations (mg/ml) were in descending order of the lines: 1, 0.4, 0.2, 0.1, 0.05 and 0.025. The insert is a plot of the reciprocals of inactivation rate constants *vs.* enzyme concentration.

Fig. 6. Relation between rate constants of inactivation and the molecular species composition of enzyme at various concentrations. Rate constants of inactivation calculated from the lines in Fig. 5 have been plotted against the relative amounts of monomer (c_1), dimer (c_2) and trimer (c_3), calculated by the method of STEINER[15] (see Table I and text).

to the origin of the coordinates suggesting that there is no inactivation when the monomer concentration is vanishingly small, *i.e.* at very high enzyme concentrations. As seen in Fig. 5, the highest rates of inactivation are recorded in dilute enzyme solutions where the monomer fractions are the largest, while the di- and trimer concentrations are relatively low.

The results of the analysis presented above suggest that dissociation of higher molecular species to the monomer precedes inactivation of the latter. In other words, we assume that in the lower range of enzyme concentrations, a rapid equilibrium is established between dimer and monomer and that inactivation of the monomer is the limiting step. In order to test this assumption, we had to show that the rate constant of the dissociation of dimer into monomer is larger than that of monomer inactivation. This proved indeed to be the case. The specific activity of glutamate dehydrogenase was shown to remain fairly constant over a wide range of enzyme concentrations[18,19], suggesting that mono-, di- and trimer have similar catalytic activities. We may state therefore that the decline in activity after a given time is proportional to the decline in the concentration of total active enzyme. We calculated the mono- and dimer distributions at the "new" concentrations by the method described above. Semilogarithmic plots of dimer and monomer concentrations were constructed against time, and first-order constants were calculated (Fig. 7). For this calculation we had to assume that the molecular weight distribution determined in phosphate buffer[17] holds not only at zero time under our conditions but also up to 0.5-h periods during which inactivation was followed. Note that the decline in concentration of both monomer and dimer follows linear (semilogarithmic) progress curves (Fig. 7). A rate constant of 0.138 min^{-1} was calculated for the dimer to monomer step and 0.069 min^{-1} for inactivation of the monomer, in the case of a 0.1 mg/ml enzyme solution. A much lower value of 0.01 min^{-1} was found for the constant calculated in a similar way for the monomer inactivation in a 1 mg/ml enzyme solution. This is not surprising if one conceives that at this concentration, higher polymers are present aside from the dimer,

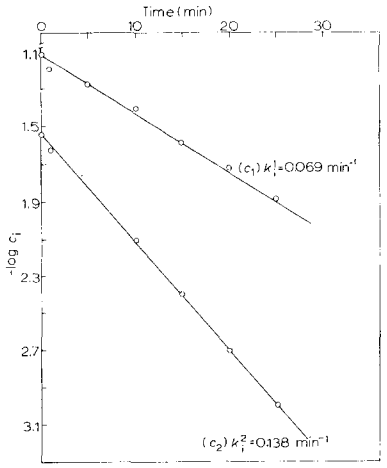


Fig. 7. Semilogarithmic plots of monomer (c_1) and dimer (c_2) concentrations calculated at various time intervals after the start of inactivation. Upper curve, monomer concentration progress curve; Lower curve, dimer concentration progress curve.

and that the continuous removal of active monomer in the course of inactivation is accompanied by dissociation of higher molecular weight species.

The above light scattering experiments[13] provide information about the molecular weight–concentration dependence for enzyme in the presence of a mixture of 1 mM GTP and 1 mM NADH. Under these conditions, the enzyme is shown to be almost completely in the monomer form. In our experiments, full stabilization was observed at concentrations of NADH above 100 μM with or without GDP, which like GTP is a dissociation promoting agent[18]. If we assume that the enzyme is dissociated, this implies that catalytically active and stable monomers (of 310 000 molecular weight) are formed in the presence of saturating amounts of NADH, which do not dissociate further. Substoichiometric amounts of reduced coenzyme, which promote inactivation, may induce a labile form of the monomer in which not all of the available sites are occupied. For this to be true, one has to assume that negative cooperative interactions exist between subunits which contain bound NADH and nonbonded subunits. The occurrence of negative homotropic interactions in NAD^+ binding to glutamate dehydrogenase has been recently reported[11]. In our experiments, addition of excess NADH to an enzyme which had been partially inactivated in the presence of low concentrations of reduced coenzyme prevents further loss of catalytic activity. In this case one may envisage the conversion of an unstable monomer with only a few sites bonded by NADH into a fully saturated and stable monomer.

ADP efficiently counteracts the inactivation promoting effect of various compounds. Incubation of a partially inactivated enzyme with ADP leads eventually to restoration of the original activity. The results obtained with addition of ADP at an advanced stage of inactivation suggest that irreversible denaturation of an inactive intermediate takes place.

In the light of our findings, 2-oxoglutarate prevents or at least slows down monomer inactivation. This is in contradiction with the previously reported lack of binding of 2-oxoglutarate in the absence of cofactor[20]. We do not know as yet in what way 2-oxoglutarate influences the state of association of the enzyme. Molecular weight determinations at various enzyme concentrations in the presence of 2-oxoglutarate should answer this problem.

ACKNOWLEDGMENTS

Our thanks are due to the Netherlands Foundation for Chemical Research (SON) which subsidized part of the investigations with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). We wish to thank the Royal Netherlands Fermentation Industries Ltd., Delft, for their support.

REFERENCES

1 M. INAGAKI, *J. Biochem. Tokyo*, 46 (1959) 893.
2 C. FRIEDEN, *J. Biol. Chem.*, 238 (1963) 146.
3 S. GRISOLIA, M. FERNANDEZ, R. AMELUNXEN AND C. L. QUIJADA, *Biochem. J.*, 85 (1962) 568.
4 G. DI PRISCO AND H. J. STRECKER, *Biochim. Biophys. Acta*, 122 (1966) 413.
5 S. GRISOLIA, *Physiol. Rev.*, 44 (1964) 657.
6 S. GRISOLIA, *Biochem. Biophys. Res. Commun.*, 32 (1968) 56.
7 C. TANFORD, *Advan. Protein Chem.*, 23 (1968) 122.
8 B. EISENKRAFT AND C. VEEGER, *Biochim. Biophys. Acta*, 167 (1968) 227.

9 J. A. OLSON AND C. B. ANFINSEN, *J. Biol. Chem.*, 197 (1952) 67.
10 K. L. YIELDING AND B. B. HOLT, *J. Biol. Chem.*, 242 (1967) 1079.
11 K. DALZIEL AND P. C. ENGEL, *Federation European Biochem. Socs. Letters*, 1 (1968) 349.
12 M. JOLY, *A Physicochemical Approach to the Denaturation of Proteins, Mol. Biol. Intern. Ser.*, Academic Press, New York, 1965, p. 197.
13 H. EISENBERG AND G. M. TOMKINS, *J. Mol. Biol.*, 31 (1968) 37.
14 H. SUND AND W. BURCHARD, *European J. Biochem.*, 6 (1968) 202.
15 R. F. STEINER, *Arch. Biochem. Biophys.*, 39 (1952) 333.
16 H. FUJITA, *Mathematical Theory of Sedimentation Analysis*, Academic Press, New York, 1962, p. 199.
17 C. FRIEDEN, *J. Biol. Chem.*, 237 (1962) 2396.
18 C. FRIEDEN AND R. F. COLMAN, *J. Biol. Chem.*, 242 (1967) 1705.
19 H. F. FISHER, D. G. CROSS AND L. L. MCGREGOR, *Nature*, 196 (1962) 895.
20 H. F. FISHER, *J. Biol. Chem.*, 235 (1960) 1830.
21 M. INAGAKI, *J. Biochem. Tokyo*, 46 (1959) 1001.