Phytochemistry, 1980, Vol. 19, pp. 11–13. Pergamon Press Ltd. Printed in England.

DEVIATIONS FROM MICHAELIS–MENTEN BEHAVIOUR OF PLANT GLUTAMATE DEHYDROGENASE WITH AMMONIUM AS VARIABLE SUBSTRATE

E. PAHLICH and CHR. GERLITZ

Institut für Allgemeine Botanik und Pflanzenphysiologie der Justus Liebig-Universität, Bereich Pflanzenphysiologie, Heinrich Buff – Ring 54–62, D 6300 Giessen, W. Germany

(*Revised received* 23 *May* 1979)

Key Word Index—*Pisum sativum*; Leguminosae; pea; glutamate dehydrogenase; stopped flow kinetic analysis; negative cooperativity; ammonium; reductive amination reaction.

Abstract—A stopped flow kinetic analysis has been performed with a homogeneous protein fraction of plant glutamate dehydrogenase. The enzyme exerts strong negative cooperativity with ammonium as variable substrate. The limiting initial rate constants for low substrate concentrations, as calculated from the kinetic data, indicate that the catalytic efficiency of the enzyme increases at low ammonium concentrations. From this it becomes evident that the reductive amination reaction is highly adaptive to the ammonium environment.

INTRODUCTION

Since the demonstration of glutamate dehydrogenase (GDH) in plants [1], the reaction of this enzyme was thought to be the primary step for ammonium assimilation (for review, see [2]). The discovery of glutamate synthetase (GS) in plants, however, led to the formulation of another mechanism for this pathway: NH_4^+ assimilation proceeds via glutamine formation by GS. In a second step glutamate is built from glutamine and 2-oxoglutarate catalysed by glutamine:2-oxoglutarate aminotranferase (GOGAT) (for review, see [3, 4]). This alternative pathway is thought to be the favoured process for NH_4^+ assimilation because of the lower K_m value for NH_4^+ and the low NH_4^+ availability in plant cells [3]. However, GDH seems to be ubiquitous in the plant kingdom and the question arises as to what physiological function it fulfils. Recently it was shown that GDH from pea seeds has a specific activity for the reductive amination reaction, which is of an order of magnitude higher than described in the literature, i.e. *ca* 500 IU/mg (Kindt, R. and Pahlich, E., unpublished results). From this and the wide distribution of the enzyme, one might expect it to be involved in the primary step of NH_4^+ assimilation.

The experiments described in this paper were performed in order to revise the K_m values for NH_4^+. In steady state kinetic experiments a marked negative cooperative effect has been observed with NH_4^+ as variable substrate. The results show that glutamate dehydrogenase can be adapted to low NH_4^+ concentrations by negative cooperativity without losing catalytic efficiency.

RESULTS AND DISCUSSION

Figure 1 shows the activity profile of plant GDH with NH_4^+ as variable substrate. With the assay conditions used here the activity is strongly inhibited by NH_4^+ concentrations greater than 125 mM. Furthermore, a faint discontinuity in the activity profile can be observed in the range 10 mM NH_4^+. A linear transformation of this data performed according to the procedure of Hanes [5] shows biphasic kinetic (Fig. 2). The corresponding K_m values (intercept with the x-axis) differ by a factor of 25 at high and low NH_4^+ concentrations (76 and 3 mM). Concomitantly the V_{max} values, as calculated from the respective activities in U/ml (reciprocal of the slope), decrease from 1.6 to 0.198 mol/min/l. From these data, limiting initial rate constants for low substrate concentrations may be calculated [5, 6] according to the expression

$$v = \frac{V_{max}}{K_m} \cdot S = k \cdot S \quad [7].$$

This calculation is possible because the experiments have been performed with a homogeneous enzyme preparation and activities arising from impurities or multiple molecular forms of the enzyme can be excluded. The respective constants k are 21 and 66 min^{-1} for high and low NH_4^+ concentrations. It can be seen that the catalytic efficiency of plant GDH increases at low NH_4^+ concentrations. From formal grounds this kinetic pattern might be explained by negative cooperativity. The characteristic feature of negative cooperativity is a decrease of both K_m and V_{max} values at lower substrate concentrations. This is valid for our observations. It is interesting to note that this type of cooperativity has also been observed with beef liver GDH. This has been done by binding measurements with coenzyme and effector molecules [8–10] as well as by kinetic studies [11]. The meaning of negative cooperativity is 'to make the reaction rate continuously responsive to changes in concentration over a

very wide range' [11], i.e. to adapt the enzyme to the respective substrate concentration.

The observed two phase pattern might also be explained in terms of half-of-the site reactivity which is the extreme consequence of negative cooperativity. This type of cooperativity is also demonstrated with the beef liver enzyme (NAD^+ as variable substrate). The effect is thought to be the consequence of a trimer–trimer association of the monomer [10, 12]. From cross-linking experiments with the plant GDH, no evidence was found that this protein also has a trimer–trimer structure (Kindt, R. and Pahlich, E., unpublished results). The underlying mechanism of the described deviation from Michaelis–Menten kinetics of plant GDH still remains to be elucidated although the data are strong evidence for negative cooperativity. The question whether NH_4^+ acts directly as an effector molecule or exhibits its function in a more general 'alloplastic' mode [13] can only be answered by binding studies. It is known, however, that plant GDH is a very flexible molecule which is easily altered by anions [14]. The results show that the kinetic behaviour of plant GDH will have to be re-investigated under appropriate experimental conditions. From the results obtained so far it becomes clear, however, that 'unphysiological' NH_4^+ concentrations are not a prerequisite of the reductive amination reaction. The assumed negative cooperativity provides a mechanism which enables the enzyme to adapt to the NH_4^+ environment without losing catalytic efficiency. Sufficient catalytic efficiency, however, is a basic requirement irrespective of the assumed function of this enzyme as a 'steady-state catalyst' (NAD^+/NADH steady-state) or as a regulatory system (NH_4^+-assimilation).

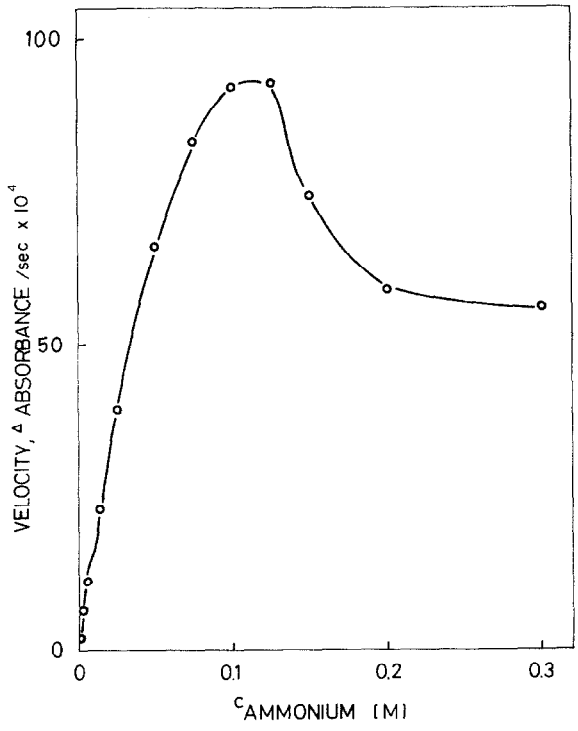

Fig. 1. Activity profile of highly purified glutamate dehydrogenase from pea seeds with NH_4^+ as variable substrate. Assay mixture as described in Experimental. Dual wavelength scan: λ measure = 340 nm, λ reference = 380 nm. Range: 0–0.1 A, $T = 23°$. Light path 2 mm. Each point is the mean value of 4 measurements. Starting with the point at high NH_4^+ concentration (0.3 M) the standard deviations (s) for the plotted mean values are: 2.1−, 2.1−, 1.3−, 2.3−, 1.7−, 1.7−, 2.5−, 0.99−, 0.7−, 0.55−, 0.17− and 0.31×10^{-4}.

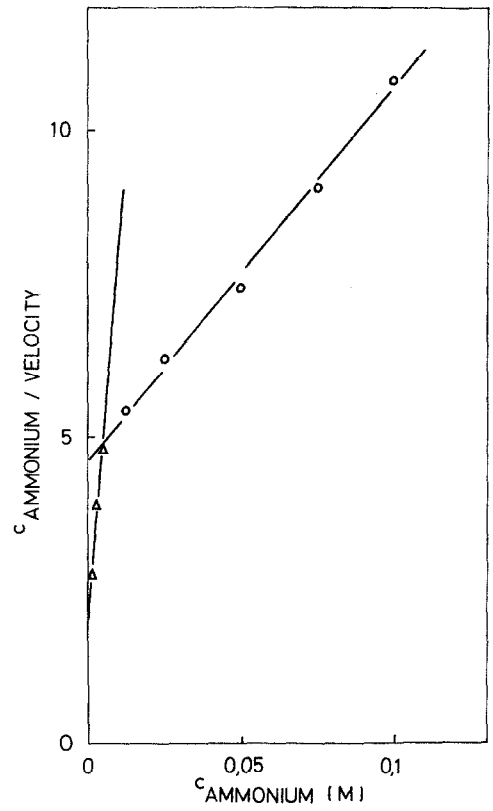

Fig. 2. Linear transformation of the data of Fig. 1 according to the Hanes plot. K_m = intercept with the x-axis, $1/V_{max}$ = slope.

EXPERIMENTAL

GDH was purified to homogeneity as described in ref. [5]. Kinetic expts have been performed with a UV-300 spectrophotometer (Shimadzu) equipped with a stopped flow device. Reservoir I contained enzyme and substrates other than NH_4^+. The concns were: 2-oxoglutarate, 28.6 mM; NADH, 357 μM; $CaCl_2$, 100 μM; enzyme, 3 μg in 0.1M Tris–HOAc buffer, pH 8 in a final vol. of 1.4 ml. Reservoir II contained NH_4 in concns ranging from 0.6M to 1×10^{-3} M in 0.1 M Tris–HOAc buffer pH 8 and a final vol. of 1.4 ml. Temp. = 25°. The reaction was started by mixing the solns from reservoirs I and II in equal amounts. Initial velocities were taken from the records after a dead time of *ca* 100 msec.

Acknowledgements—We thank Kontron Company, for the opportunity to perform the stopped flow measurements.

REFERENCES

1. Bulen, W. A. (1956) *Arch. Biochem. Biophys.* **62,** 173.
2. Sims, A. P., Folkes, B. F. and Bussey, A. H. (1968) *Recent Aspects of Nitrogen Metabolism in Plants.* (Hewitt, E. J. and Cutting, C. V., eds.) p. 91. Academic Press, London.
3. Miflin, B. J. and Lea, P. J. (1977) *Annu. Rev. Plant Physiol.* **28,** 299.
4. Miflin, B. J. (1976) *Phytochemistry* **15,** 873.
5. Wong, J. T.-F. (1975) *Kinetics of Enzyme Mechanism*, p. 9. Academic Press, London.
6. Laidler, K. J. (1958) *Chemical Kinetics of Enzyme Action.* Clarendon Press, Oxford.
7. Casey, E. J. and Laidler, K. J. (1950) *J. Am. Chem. Soc.* **72,** 2159.
8. Koberstein, R. and Sund, H. (1973) *Eur. J. Biochem.* **36,** 545.
9. Pantaloni, D. and Dessen, P. (1969) *Eur. J. Biochem.* **11,** 510.
10. Sund, H., Dieter, H., Koberstein, R. and Rasched, J. (1977) *J. Mol. Catal.* **2,** 1.
11. Engel, P. C. and Dalziel, K. (1969) *Biochem. J.* **115,** 621.
12. Sund, H., Markau, K. and Koberstein, R. (1975) in *Subunits in Biological Systems* (Timasheff, S. N. and Fasman, G. D., eds.) p. 226. Marcel Dekker, New York.
13. Klotz, J. M. (1966) *Arch. Biochem. Biophys.* **116,** 92.
14. Pahlich, E., Gelleri, B. and Kindt, R. (1978) *Planta* **138,** 161.