PHENYLALANINE AMMONIA-LYASE: AN UPDATE ON ITS KINETICS

NAYEEM ULLAH KHAN, MANZOOR AHMAD BHAT* and C. S. VAIDYANATHAN†
Department of Botany, St Joseph's College, PG Centre, Bangalore 560 001, India.
**Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India.*

ABSTRACT

Phenylalanine ammonia-lyase is a key enzyme in the phenolic metabolism of plants. Aspects of kinetics of this enzyme are reviewed. Of the several models, the partially concerted model of subunit interactions accounts most fully for its kinetic behaviour. A form of cucumber PAL reverses the usual reaction catalysed by the enzyme under the standard assay conditions. The partially concerted model has been applied to explain the kinetics of this new form of PAL.

INTRODUCTION

PHENYLALANINE ammonia-lyase (EC 4.3.1.5) (PAL) is an intensively studied enzyme of plant phenylpropanoid metabolism. It is one of the most difficult enzymes to purify[1]. Affinity procedures for its purification are difficult[2] to reproduce. It has been the subject of many recent reviews[1-3], which, however, have not covered its kinetic properties and their relationship to affinity purification. The enzyme has potential use in cancer therapy[4] and finds application in combating genetic disorders like phenylketonuria[5]. It could be exploited for the industrial production of L-phenylalanine from *trans*-cinnamic acid. The amino acid is in great demand for the industrial production of Aspartame, a peptide artificial sweetener[6].

PAL from many sources shows subtle kinetic behaviour. Most sources yield enzyme preparations that show negative co-operativity[7]. The reasons for the deviant and often anomalous kinetic properties of PAL are far from clear. One of the objectives of the present article is to discuss some of them in detail. Also discussed here are some interesting and unusual kinetic properties of PAL from cucumber seedlings, observed for the first time in our laboratory[8]. The partially concerted model proposed by Ricard *et al*[9] has been presented in a modified form to explain the unexpected and novel catalytic behaviour of cucumber PAL.

CHARACTERISTICS OF NEGATIVELY CO-OPERATIVE ENZYMES

Negatively co-operative enzymes show the following properties[7]: (i) they exhibit biphasic saturation kinetics; (ii) Hofstee–Eadie plots are concave; (iii) Lineweaver–Burk plots are straight lines often bending downwards at the origin; (iv) the R_S value (ratio of substrate concentrations required to achieve 90% and 10% of V_{max}) is greater than 81; (v) the Hill co-efficient is less than one. Negative co-operativity can be suppressed in PAL by benzoic acid[9]. This effector can even reverse the co-operative effects exhibited by the enzyme. Surprisingly, PAL from soybean shows positive co-operativity after certain treatments.

SIGNIFICANCE OF CO-OPERATIVE INTERACTIONS IN PAL

Conway and Koshland[10], using equilibrium dialysis, determined the binding constants for NAD for the enzyme glyceraldehyde-3-phosphate dehydrogenase. They isolated complexes ED_1, ED_2, ED_3 and ED_4, which had 1–4 moles of NAD bound per mole of enzyme. The increase in the binding constant was an order of magnitude greater for every molecule of NAD bound to the enzyme. Binding of one molecule of NAD decreased binding of subsequent molecules of ligand to the enzyme—this is negative co-operativity.

It has been demonstrated by Cornish-Bowden[11] that the more negatively co-operative a system is, the less sensitive it is to relative changes in ligand concentration at all values of fractional saturation of the system. This can be visualized as maintaining constant levels of activity over a wide range of substrate concentration. Enzymes which bind substrate tightly would achieve the same effect without the need for negative co-operativity. This is the case with mammalian hexokinases, which do not exhibit

†For correspondence.

negative co-operativity and which nevertheless are insensitive to changes in glucose concentration in the near-physiological range[12].

Lamb and Rubery[13] have extended the analysis of Cornish–Bowden to consider the effect of substrate analogues as a function of co-operativity of the binding system. These authors have come to the conclusion that the effect of negative co-operativity is to make an enzyme more sensitive to inhibition by relative increases in the concentration of substrate analogues (product) at the expense of sensitivity to relative changes in substrate concentration compared to positively co-operative and non-cooperative systems.

PAL, being the first enzyme of phenylpropanoid metabolism, must be sensitive to events in the phenylpropanoid pathway but less sensitive to changes in substrate concentration. Alteration in phenylalanine concentration may be brought about by channelling this metabolite into protein synthesis or by changes in the rate of its biosynthesis. The biosynthesis of phenylalanine occurs via the shikimic acid pathway, whose first enzyme 3-deoxy-D-arabinose heptulosonic acid-7-phosphate synthetase is insensitive to L-phenylalanine in higher plants[13].

The antithesis of negative co-operativity is positive co-operativity, where the enzyme is less sensitive to relative changes in product concentration but more sensitive to changes in substrate level. PAL from soybean exhibits positive co-operativity depending on the conditions of isolation[14], whereas maize PAL loses co-operativity completely on storage at $-10°C$ for a year[15]. Such changes in co-operativity may not be of physiological importance to plants but may be important for employing the enzyme to manage phenylketonuria[16] or for using the enzyme to determine L-phenylalanine in serum samples[17]. All these studies show that a thorough knowledge of the fundamental aspects of PAL is necessary for a better understanding of its applications.

MODELS FOR CO-OPERATIVE INTERACTIONS

PAL has four subunits[2]. There seem to be two active sites per molecule of the enzyme[2]. The concept of subunit interaction is most useful for understanding the molecular basis of enzyme regulation. Three types of models have been postulated. The allosteric model of Monod *et al*[18] emphasizes the idea of pre-equilibrium between conformational states with symmetry conservation. The sequential models are based on induced fit[19]. The flip-flop model[20] implies reactivity of half of the sites on the enzyme molecule. The interpretation of kinetic data for PAL is complicated because the existing models deal with polymeric enzymes which act on more than one substrate. A detailed mathematical account of the structural rate equation for both dimeric and tetrameric states has appeared in the literature. This is the model of Ricard *et al*[9] and is known as the partially concerted model. Earlier models of co-operativity predicted a lag phase in the catalytic reaction; the partially concerted model predicts both lag and bursts. Such bursts in the release of products of PAL have been observed by fast-kinetic methods[21].

The model discussed above is shown in figure 1 (unhatched portion). The conformational changes in the model are as follows. One or two molecules of the substrate can bind to the dimeric enzyme. When both the molecules bind to the enzyme the two subunits are the conformation represented by squares.

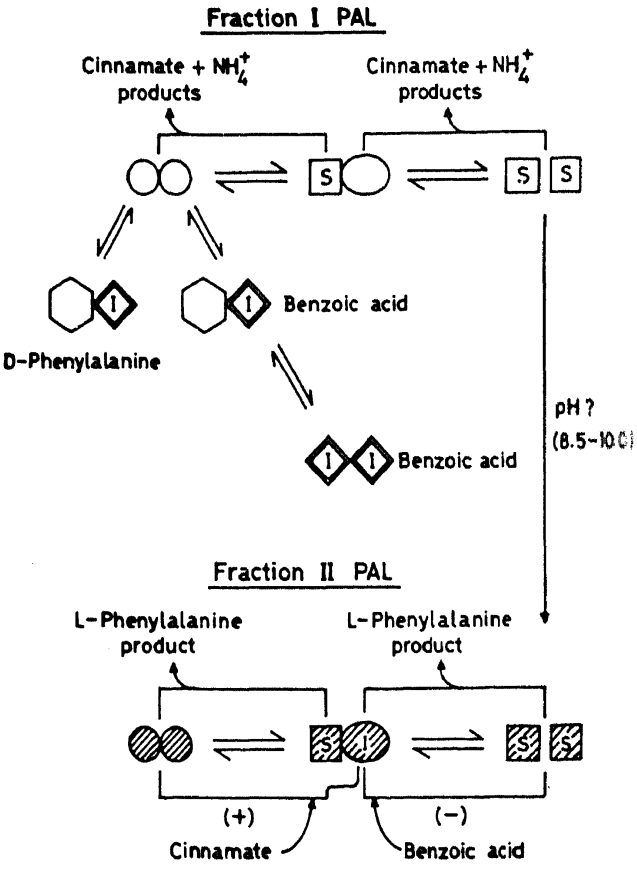

Figure 1. A model to explain kinetic behaviour of cucumber PAL. [S, Substrate (L-phenylalanine or *trans*-cinnamic acid); I, Inhibitor (D-phenylalanine or benzoic acid.)]

in the figure. On conversion of one molecule of substrate to product, the enzyme attains a hybrid conformation. In the hybrid state half of the enzyme conserves the square conformation, while the other half attains a totally new conformation (ellipsoid). The square conformation releases the product and the entire dimer is now in another conformation (circles), which can change to a new hybrid state (rhombus and hexagon). However, the dimer in this state does not conserve any part of the original conformation (circle) from which it is derived. Kinetic studies show that this totally new hybrid conformation can take part in two reaction pathways. In one pathway the enzyme can bind one molecule of inhibitor like D-phenylalanine; in another it can bind two molecules of inhibitor like benzoic acid.

APPLICATION OF PARTIALLY CONCERTED MODEL TO PAL FROM CUCUMBER SEEDLINGS

Cucumber PAL elutes from the affinity column in two fractions. One fraction (fraction 1 PAL) shows typical biphasic kinetics as shown by wheat PAL. Hence the behaviour of fraction 1 can be represented by the partially concerted model discussed above. Fraction II PAL of cucumber totally reverses the reaction. It converts *t*-cinnamate to L-phenylalanine in the presence of NH_4^+ ions. Like fraction I PAL, fraction II PAL can bind two molecules of substrate (*t*-cinnamate). This model predicts abolition of negative co-operativity. The model differs slightly from the partially concerted model in that the partially conserved hybrid conformation can bind to an inhibitor molecule which can be *t*-cinnamate (substrate), or an inhibitor like benzoic acid, which can almost block the forward reaction of PAL. In the presence of benzoic acid fraction II PAL must be efficient in converting cinnamate to L-phenylalanine. Experimentally this is found to be the case. However, the model does not explicitly formulate the release of ammonia. The hatched portion of figure 1 shows the model for fraction II PAL from cucumber. The change from fraction I PAL to fraction II PAL may be brought about by pH. At the moment the nature of this transition is not clear. However, it must be noted that earlier attempts to reverse the reaction of PAL met with only partial success[22]. In yeast cells reversibility was achieved only recently[5, 6]. It is possible that in yeast also there is a form of PAL which reverses the reaction.

CONCLUSION

To conclude, it can be said that of the various models discussed, the best model to explain the kinetic behaviour of PAL is the partially concerted model of Ricard *et al*[9]. This model can partly accommodate the kinetics of a totally new form of PAL from cucumber seedlings. The new behaviour of the enzyme was detected with a spectrophotometer with an attached computer. The continuous spectrophotometric assay was used to monitor fast kinetics[23]. The spectrophotometric assay developed in this laboratory is suitable for crude preparations[24, 25]. It appears that the purified preparation of PAL from cucumber can be assayed only by a spectrophotometer with fast-kinetics facility. The reversible reaction shown by the cucumber enzyme could be employed in the production of L-phenylalanine from cinnamate and NH_4^+ ions. We have used partially purified preparations of PAL to monitor changes in L-phenylalanine in rat plasma[26]. Crude preparations of the enzyme make PAL-linked spectrophotometric estimation of L-phenylalanine a possibility. Further the reversibility of cucumber PAL may have a physiological significance. It has been shown that cinnamate is toxic to cucumber seedlings[27]. It is perhaps for this reason that cinnamate is never released in cucumber cotyledons. It is converted immediately to *p*-coumarate by cinnamate 4-hydroxylase (CA_4H). In cucumber, PAL and CA_4H are tightly coupled enzymes in microsomes[28]. Even if, by some physiological event, these coupled enzymes become separated (ethylene can uncouple them), the uncoupled PAL with changed properties can convert cinnamate back to L-phenylalanine. This can happen at a slower rate since relatively high concentrations of L-phenylalanine can marginally inhibit PAL but are not toxic to plants. Further work on PAL isoenzymes is needed to fully understand the model and kinetic mechanisms proposed in this review. Since monoclonal antibodies are available[29], highly purified preparations of PAL would be easier to obtain by immunoaffinity procedures. Many more isoenzymes may be revealed by isoelectric focusing[30].

ACKNOWLEDGEMENT

Financial assistance from the Indian Institute of Science is gratefully acknowledged.

1 July 1988; Revised 7 September 1988

1. Jones, D. H., *Phytochemistry*, 1984, **23**, 1349.
2. Hanson, K. R. and Havir, E. A., In: *The biochemistry of plants–A comprehensive treatise*, (eds) P. K. Stumpf and E. E. Conn, Academic Press, New York, 1981, Vol. 7, p. 577.
3. Abell, C. W. and Shen, R. S., *Methods Enzymol.*, 1987, **142**, 243.
4. Abell, C. W., Hodgins, D. S. and Stith, W. J., *Cancer Res.*, 1973, **33**, 2529.
5. Hoskins, J. A., Pieris, J. D., Starr, D. J. T., Wade, H. D., Wright, E. C. and Stin, J., *Lancet*, 1980, **1**, 392.
6. Evans, C. T., Conrad, D., Danna, K., Peterson, W., Choma, C. and Misawa, M., *Appl. Microbiol. Biotechnol.*, 1987, **25**, 399.
7. Iredale, S. E. and Smith, H., *Phytochemistry*, 1974, **13**, 575.
8. Bhat, M. A., Khan, N. U. and Vaidyanathan, C. S., unpublished results.
9. Ricard, J., Mouttet, Ch. and Nari, J., *Eur. J. Biochem.*, 1974, **41**, 479.
10. Conway, A. and Koshland, D. E. Jr., *Biochemistry*, 1968, **7**, 4011.
11. Cornish-Bowden, A., *J. Theor. Biol.*, 1975, **51**, 233.
12. Walker, D. G., *Essays Biochem.*, 1966, **2**, 223.
13. Lamb, C. J. and Rubery, P. H., *J. Theor. Biol.*, 1976, **60**, 441.
14. Havir, E. A., *Arch. Biochem. Biophys.*, 1981, **211**, 564.
15. Hanson, K. R., *Arch. Biochem. Biophys.*, 1981, **211**, 575.
16. Marconi, W., Bartoli, F., Granna, R., Morisi, F. and Sportono, G., *Biochemie*, 1980, **62**, 557.
17. Shen, R. S. and Abell, C. W., *Science*, 1977, **197**, 665.
18. Monod, J., Wyman, J. and Changeaux, J. P., *J. Mol. Biol.*, 1961, **12**, 88.
19. Koshland, D. E. Jr., Nemethy, G. and Filmer, D., *Biochemistry*, 1966, **5**, 565.
20. Bernhard, S. A., Dunn, M. F., Luisi, P. L. and Schack, J., *Biochemistry*, 1970, **9**, 185.
21. Richard, J., Nari, J., Buc, J. and Meunier, J.-C., In: *Regulation of enzyme synthesis and activity in higher plants*, (ed.) H. Smith, Academic Press, New York, 1977, p. 156.
22. Havir, E. A. and Hanson, K. R., *Biochemistry*, 1968, **7**, 1896.
23. Zucker, M., *Plant Physiol.*, 1965, **40**, 779.
24. Khan, N. U. and Vaidyanathan, C. S., *Curr. Sci.*, 1986, **55**, 391.
25. Khan, N. U. and Vaidyanathan, C. S., *Biochem. Int.*, 1987, **14**, 451.
26. Khan, N. U., Bhat, M. A., Vaidyanathan, C. S., unpublished results.
27. Khan, N. U. and Vaidyanathan, C. S., *Plant Soil*, 1987, **97**, 299.
28. Czichi, U. and Kindl, H., *Planta*, 1977, **134**, 133.
29. Bolwell, G. P., *Phytochemistry*, 1988, **27**, 1235.
30. Belumis, C. J. and Hrazdina, G., *Phytochemistry*, 1988, **27**, 2485.

ANNOUNCEMENT

DIRECTORY OF ENVIRONMENTAL CHEMISTRY GROUPS IN INDIA

During the deliberations of the International Seminar on Analytical Techniques in Monitoring the Environment (ISAME 89) held recently, it was proposed to take up a Co-ordinated Programme for Monitoring the Environment. To begin with it is proposed to prepare a Directory of Environmental Chemistry Groups in India. Preparation of this directory is now in progress. Laboratories, departments and scientists involved in pollutant research and/or environmental chemistry may write to Prof. S. Jayarama Reddy, Department of Chemistry, S. V. U. College of Engineering, Tirupati 517 502, before 30 April 1989 for a blank form.

The national co-ordinating committee will select a few of the most polluting agents and arrive at standardized procedures for each. The various participating groups can then monitor pollutants in their regions. The data will then be analysed and submitted to government agencies responsible for the environment.