raised by 2°, reflecting the well-known stabilization of creatine kinase by its substrates (Kuby, Noda & Lardy, 1954b).

Alkylation of the catalytic-site thiol groups of creatine kinase with iodoacetate almost completely abolishes the second-order transition (Fig. 1, *C*) but this is considerably restored by the addition of substrate (Fig. 1, *D*). The peak of the denaturation endotherm is lowered to 58.5° by carboxymethylation but again substrates protect to the extent of 2°.

When iodoacetamide is used as the alkylating agent instead of iodoacetate the thermogram for the enzyme alone is markedly altered (Fig. 1, *E*). The second-order transition is completely abolished and the enzyme becomes very unstable, with the peak of the denaturation endotherm being lowered to 53.5°. Addition of substrates raises the endotherm peak to 57° but does not restore the transition (Fig. 1, *F*).

These results clearly show that maintenance of a negative charge on the reactive thiol group is essential for the maintenance of enzyme conformation and stability. That charge is the most important factor is revealed by the finding that iodoacetate has a relatively small effect as compared with iodoacetamide. However, although carboxymethylated creatine kinase will bind substrates with a change in the second-order transition and an increase in stability similar to that found with the native enzyme, it is nevertheless catalytically inert. This suggests that control of conformation and protein stability by the judicious location of a negative charge is not the sole function of the reactive thiol group and it seems reasonable to infer that it must also participate in the catalytic reaction.

In the light of the present findings the mechanism of action proposed by Watts & Rabin (1962) also offers a tentative explanation for the basis of the conformational change. As creatine binds to the

enzyme the guanidino group is brought in close proximity to the reactive thiol group. Interaction between thiol group and guanidino group is promoted by the binding of the ATP-Mg²⁺ complex that eliminates the negative charge on the thiol group and induces the conformational change. In support of this hypothesis O'Sullivan & Cohn (1966) have reported proton relaxation-rate studies on native and alkylated enzyme from which they infer an interaction between creatine and the catalytic-site thiol group. There is no evidence about how blocking of the negative charge induces the conformational change in creatine kinase, but Watts & Rabin (1962) have inferred on the basis of the effect of ionic strength on the rate of alkylation by iodoacetate the presence of two positive charges close to the reactive thiol group that could possibly be involved in this process.

It is clear that a purely chemical role is inadequate to account for the function of the essential thiol group in the mechanism of action of creatine kinase *in vitro* and that it must also be involved in producing the associated conformational changes.

The author is grateful to the DuPont Co. (U.K.) Ltd. for providing the Differential Thermal Analyser and to Mr I. C. Wylie of the DuPont Co. for operating it. It is also a pleasure to thank the Biochemistry Department of University College London where this work was carried out.

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Biochem. J. (1966) **100**, 14c

Mevalonate-Kinase Isoenzymes in Plant Cells

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(Received 18 May 1966)

A scheme for the regulation of terpenoid biosynthesis in developing seedlings by a combination of intracellular enzyme segregation and specific

membrane permeability has been proposed (Goodwin & Mercer, 1963; Treharne, Mercer & Goodwin, 1966). The basis of this scheme is that

enzymes for the conversion of MVA* into farnesyl pyrophosphate exist both inside and outside the chloroplast, though the chloroplast membrane is itself impermeable to the substrates and products of the biosynthetic sequence. In support of this scheme it has been shown (Rogers, Shah & Goodwin, 1966) that mevalonate kinase and phosphomevalonate kinase, enzymes catalysing reactions (1) and (2) respectively, are present in

chloroplasts and that the chloroplast membrane is essentially impermeable to MVA.

Further studies have now shown that mevalonate-activating enzymes are present both inside and outside the chloroplast and this work is the subject of the present communication.

Plant material. The source of plant material used throughout the investigation was *Phaseolus vulgaris* (French-bean) seedlings.

Isolation of chloroplasts. Intact chloroplasts were prepared as described previously (Rogers *et al.* 1966) by a discontinuous-density-gradient centrifugation technique with non-aqueous solvents.

Acetone-dried powders. Acetone-dried powders of etiolated cotyledons of young dark-grown French-bean plants were prepared by first vigorously homogenizing small pieces of cotyledon in acetone at -10° with an Ultra-Turrax homogenizer. The white powder was filtered off and dried at room temperature before storage at -10° in tightly capped jars until use.

Leaf tissue. Before extraction of enzyme green leaves were cut into small pieces, then frozen and freeze-dried. Etiolated leaves were deveined but not freeze-dried.

Extraction of enzymes. The extraction of soluble enzymes from chloroplasts and acetone-dried powders was achieved by suspension of the preparations in 3mM-KH₂PO₄-Na₂HPO₄ buffer, pH 7.1 (95mg. of chloroplasts/1.3ml. of buffer; 250mg. of acetone-dried powder/2.5ml. of buffer). Extraction of enzymes was aided by ultrasonic treatment of the chloroplast suspension and by stirring the acetone-dried powder suspension.

Homogenates of etiolated leaves of French-bean plants were obtained by grinding small pieces of leaf tissue in a cold mortar with sand and 0.40M-sucrose-0.01M-potassium phosphate buffer, pH 7.4 (6.0g. of tissue/6.0ml. of buffer). Freeze-dried green leaves were homogenized in 3mM-phosphate buffer, pH 7.1, with the Ultra-Turrax homogenizer

* Abbreviations: MVA, mevalonic acid; MVA-5P, mevalonic acid 5-phosphate; MVA-5PP, mevalonic acid 5-pyrophosphate.

(700mg. of freeze-dried tissue/10.0ml. of buffer). Inactivation of enzymes by tannins and phenolic compounds in the leaves was avoided by the addition of 0.6g. of Polyclar AT/g. of freeze-dried tissue or 1.0g. of Polyclar AT/g. of fresh tissue (Loomis & Battaile, 1966; Sanderson, 1966) during grinding. Cell debris was removed by filtration through several layers of muslin and the suspension of cell particles was centrifuged at 30000g for 30min. at 2° . The supernatant fraction was used in subsequent investigations.

Detection and estimation of enzyme activity. The method used to detect and assay enzyme activity was, in principle, that described previously (Rogers *et al.* 1966). The assay medium contained (per tube): buffer salts, 50 μ moles; MgSO₄, 1.5 μ moles; GSH, 6.0 μ moles; ATP, 6.0 μ moles; DL-[2-¹⁴C]-MVA, 1.5 μ moles (26100 disintegrations/sec.). The final volume per incubation tube was 0.5 ml. before the addition of 0.1 ml. of enzyme extract containing 1–3mg. of protein. This addition caused negligible change in the selected pH of the assay mixture, which was checked before and after the incubation. The effect of pH on the activity of mevalonate kinase was studied over the range pH 4.0–9.0 with the buffers described in the legend to Fig. 1. Incubations were at 36° and for times such that the conversion of MVA into MVA-5PP was still proceeding linearly with time at the end of each incubation.

Results and discussion. At the end of all incubations, even those of only a few minutes, the main product detected was MVA-5PP. This was separated from MVA and minor ¹⁴C-labelled reaction products by paper chromatography, detected on the chromatogram by radioautography and estimated by counting areas of the chromatogram in a Packard Tri-Carb scintillation counter (Rogers *et al.* 1966).

The results obtained from such incubations are shown in Fig. 1. The peak of activity in chloroplasts is at pH 7.5 whereas that in etiolated cotyledons is at pH 5.5. Calculations from results obtained after a 2hr. incubation showed the activity at pH 7.5 of the cotyledon preparation was 0.62m μ mole of MVA phosphorylated/min. Neither preparation showed activity in the other pH range if the chloroplast preparation was free of contamination by cytoplasmic proteins or if young etiolated cotyledons were used.

Etiolated and green leaves show peaks of activity at both pH 5.5 and pH 7.5, though the ratio of activity at pH 5.5 to that at pH 7.5 is greater with etiolated tissue.

The production of MVA-5PP from MVA is the result of two successive reactions, catalysed respectively by mevalonate kinase and phosphomevalonate kinase, by which MVA is converted, through

Fig. 1. Effect of pH on the activity of mevalonate kinase in preparations from French-bean seedlings: (a) chloroplasts; (b) etiolated cotyledons; (c) green leaves; (d) etiolated leaves. The buffers used were at about 0.084 M final concentration: ■, acetate-phosphate; ○, phosphate; ●, tris-HCl. The incubation times after which assays were carried out were: (a) 30 min.; (b) 20 min.; (c) 50 min.; (d) 30 min. If the ordinates are numbered from 0 to 100 the plots represent the percentage conversion of available MVA into MVA-5PP in these times at each pH.

MVA-5P, into MVA-5PP (reactions 1 and 2). The results shown in Fig. 1 can be adequately explained only by the conclusion that the pH optimum for the enzyme catalysing reaction (1) is at pH 7.5 in chloroplasts and at pH 5.5 outside the chloroplast.

Though the presence of phosphomevalonate kinase in the two sites is shown by the fact that all preparations give MVA-5PP as the main product, a similar conclusion cannot be made with regard to the behaviour at various pH values of the chloroplastidic and extrachloroplastidic phosphomevalonate kinases since the alternative possibility exists that, as in yeast, enzymic activity remains constant in the range pH 5.5–10.0 (Bloch, Chaykin, Phillips & de Waard, 1959).

No other interpretation of the results appears possible. If, for example, the activity of mevalonate kinase were constant over the range pH 5.5–7.5 whereas that of phosphomevalonate kinase showed the behaviour attributed above to mevalonate kinase, then the chloroplasts at pH 7.5 would convert MVA into MVA-5PP but at pH 5.5 MVA-5P would accumulate. In fact only insignificant quantities of MVA-5P were ever detected in any incubation mixture.

The results thus indicate the presence of two mevalonate-kinase isoenzymes in plant cells. One,

in chloroplasts, is active at pH 7.5 and shows no activity at pH 5.5, whereas the second enzyme, present extrachloroplastidically, shows the opposite behaviour. Green leaves, as one might expect, possess both enzymes and so do etiolated leaves, thus indicating the potentiality of etiolated plastids for synthesis of some, at least, of the terpenoid precursors.

Mevalonate-kinase isoenzymes with different pH optima have not been reported previously in other organisms. The pH optima of the enzymes from rabbit liver (Markley & Smallman, 1961), pig liver (Levy & Popják, 1960) and *Hevea* (Williamson & Kekwick, 1965) are all about pH 7.5, and that of yeast (Tchen, 1958) is at pH 6.4–6.7. Loomis & Battaile (1963) report that the pH optimum of mevalonate kinase in the 105000g supernatant fraction of etiolated pumpkin cotyledons is at pH 5.5 with a steady decrease in activity at higher pH values. At pH 7.5 the activity was still about half that at pH 5.5. However, their data are consistent with the presence of the two isoenzymes that have now been demonstrated in etiolated leaves of French bean (Fig. 1d).

The discovery of different mevalonate kinases catalysing the same reaction is especially noteworthy since the enzymes reside in different intra-

cellular sites. The difference in the pH optima of the mevalonate-kinase isoenzymes appears to be far greater than is usually found between isoenzymes. Though L-aspartate-2-oxoglutarate aminotransferases of mitochondria and the 100 000g supernatant of rat-liver homogenates have optima over the range pH 5.5–9.0 and at pH 8.0–9.0 respectively, the latter enzyme still shows appreciable activity (30%) at pH 5.5 (Fleischer, Potter & Wakim, 1960; Boyd, 1961). A number of acid phosphatases exist in different mammalian tissues that show pH optima varying from pH 4.0 to pH 6.0 (Moore & Angeletti, 1961). Commonly, however, pH optima of isoenzymes differ very little. Thus the three arylesterase isoenzymes in serum have optima at pH 7.9, 7.9 and 8.5 respectively (Wilde & Kekwick, 1964). For the β -glucuronidases of mitochondria (optimum pH 4.7) and microsomes (optimum pH 5.2) of rat liver the difference in optima can be attributed to combination of the microsomal enzyme with ribonucleic acids (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955).

Differences in intracellular localization of isoenzymes in higher plants have been reported but usually, as with the malate dehydrogenases of mitochondria and cell sap of pea epicotyls, the isoenzymes show almost identical properties (Davies, 1961).

The existence of mevalonate kinases inside and outside the chloroplast supports the view that there are two distinct pathways of terpenoid biosynthesis in plant cells and suggests that other enzymes in chloroplasts may show similar differences compared with enzymes catalysing the same reactions else-

where in the cell. Interpretations of results obtained with homogenates of plant cells should take into consideration the possibility of such differences, and the extrapolation of results from such preparations to situations within intracellular organelles should be made with caution.

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Biochem. J. (1966) **100**, 17c

Electrophoretic Separation of Cytoplasmic Ribonucleic Acid Fractions of Pancreas and Liver

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(Received 25 May 1966)

Electrophoretic separation of different types of RNA has been achieved by using agar or starch gels as supporting media. Bachvaroff & McMaster (1964) resolved the RNA of microsomes of liver and of lymph nodes into five electrophoretic bands by agar-gel electrophoresis. Tsanev (1965) used a similar method to separate the RNA fractions of

rat liver. Székely, Beney, Vincze, Gaál & Tamás (1966) separated the ribosomal RNA of pigeon pancreas into four main fractions and some minor components by starch-gel electrophoresis. The electrophoretic patterns of RNA prepared from different cytoplasmic fractions of liver and pancreas were compared in the present experiments and the