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PROPERTIES OF CITRATE SYNTHASE FROM *PISUM SATIVUM* MITOCHONDRIA

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Key Word Index—*Pisum sativum*; Leguminosae; pea; citrate synthase; sulphydryl groups; *p*-hydroxymercuribenzoate; 5,5′-dithiobis-(2-nitrobenzoic acid).

RESULTS AND DISCUSSION

This paper reports some properties of citrate synthase extracted from pea epicotyl mitochondria. Citrate synthase, from pea epicotyls, has as its pH optimum a value of 8.6. The enzyme displayed Michaelis–Menten kinetics for both acetyl CoA (AcSCoA) and oxaloacetate (OAA). Using a Lineweaver–Burk plot, apparent K_m values for AcSCoA and OAA were 31 and 16 μM, respectively.

One end-product of the TCA cycle, ATP, was found to be an effective inhibitor of the enzyme. At 5 mM ATP, the activity of the enzyme was inhibited by 50%. Both ADP and AMP were much less effective as inhibitors of the enzyme, e.g. at 5 mM ADP or AMP, citrate synthase was inhibited by 25 or 8%, respectively. The inhibition by ATP was found to be competitive with respect to AcSCoA and non-competitive with respect to OAA. Other nucleotides, i.e. 5 mM NAD, NADH, NADP or NADPH had no effect on enzyme activity. These results lend weight to the suggestion that citrate synthase is a regulatory enzyme [1]. Here, then, is a very plausible feedback mechanism whereby ATP, the ultimate end-product of the TCA cycle, controls the first enzyme of the pathway.

Chemical modification studies are helpful in identifying functional groups involved in the catalytic process. The effect of certain sulphydryl reagents on citrate synthase from peas was investigated. The enzyme was incubated with a number of sulphydryl reagents for various periods of time, at 0°, and then assayed for citrate synthase activity. *p*-Hydroxymercuribenzoate (pHMB) (10^{-4} M) rapidly inhibited the enzyme whereas both $HgCl_2$ (10^{-4} M) and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (10^{-3} M) had little effect. After 25 min pre-incubation with pHMB citrate synthase was inhibited by 60%. During this same period of time $HgCl_2$ inhibited the enzyme by only 7% and no inhibition was observed with DTNB. If, in addition to pHMB, the enzyme was also pre-incubated with 1 mM OAA, 91% of the activity still remained after 25 min whereas no such protection of activity was observed with pHMB and 1 mM AcSCoA. Thus, it would appear that in peas, sulphydryl groups are necessary for catalytic activity at the oxaloacetate binding site.

Work is now in progress to characterise the properties of this enzyme further in an attempt to gain an understanding of the *in vivo* role of citrate synthase in plants.

EXPERIMENTAL

Seeds of *Pisum sativum* L., cv Kelvedon Wonder were grown in vermiculite, in the dark, at 22° for 7 days. The epicotyls were then excised, washed with H_2O and immediately chilled.

Preparation and sonication of mitochondria. The procedure followed was a modified version of that described by ref. [2]. All steps were performed at 1–4°. Excised epicotyls were ground in a pestle and mortar with a buffer composed of 0.25 M Tris–HCl, 0.3 M mannitol and 1 mM EDTA pH 7.5. (1 part tissue: 2 parts buffer). The brei was filtered through 4 layers of muslin and the filtrate centrifuged at 1000 *g* for 15 min. The pellet of cell debris was discarded and the supernatant centrifuged at 10000 *g* for 15 min. The resulting pellet, containing the mitochondria, was washed in a buffer composed of 50 mM Tris–HCl, 0.3 M mannitol and 1 mM EDTA pH 7.5 and then re-centrifuged at 10000 *g* for 15 min. To the resulting mitochondrial pellet was added a small vol. of 50 mM Tris–HCl, 1 mM EDTA pH 7.5 and the suspension was then sonicated for 3 min. This suspension was centrifuged at 20000 *g* for 10 min, the resulting pellet being discarded and the supernatant retained for further treatment.

Protamine sulphate and ammonium sulphate fractionation. Protamine sulphate was added to the supernatant slowly, with stirring, for 15 min at a concn of 1 mg/10 mg protein. Centrifugation at 22000 *g* for 20 min pelleted the precipitate. This was then discarded. Solid ammonium sulphate was added to the supernatant with stirring (20 min) to give 45% saturation and then centrifuged at 22000 *g* for 20 min. The pellet was discarded and the supernatant brought to 70% saturation with ammonium sulphate, stirred for 20 min and re-centrifuged at 22000 *g* for 20 min. A small vol. of 20 mM Tris–HCl, 1 mM EDTA pH 7.5 (*ca* 1 ml) was added to the resulting pellet. This suspension was then desalted through G-25 fine Sephadex. At this point the enzyme was stable at 2° for a few days.

Assay of citrate synthase. Citrate synthase was assayed spectrophotometrically at 412 nm at 25° by the method of ref. [3]. Assay mixtures contained 0.1 M Tris–HCl (pH 8.6), 1 mM EDTA, 0.1 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 0.2 mM acetyl CoA, 0.2 mM freshly prepared oxaloacetate and enzyme in a total vol. of 1.0 ml. Acetyl CoA was prepared by acylation of CoASH with Ac_2O essentially according to ref. [4]. Protein determinations were according to the method of ref. [5].

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