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INHIBITION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE BY 2-OXOACIDS

TERESA ITURRIAGAGOITIA-BUENO, EDWARD J. GIBSON,* CHRISTOPHER J. SCHOFIELD* and PHILIP JOHN†

Department of Agricultural Botany, School of Plant Sciences, Plant Science Laboratories, The University of Reading, Reading RG6 6AS, U.K.; *Dyson Perrins Laboratory and the Oxford Centre for Molecular Sciences, University of Oxford, South Parks Road, Oxford OX1 3QY, U.K.

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Key Word Index—*Pyrus communis*; Rosaceae; pear fruits; ACC oxidase; 2-oxoacids; dioxygenase.

Abstract—An extract from ripe pear fruits was used to demonstrate that 1-aminocyclopropane-1-carboxylate (ACC) oxidase, the enzyme responsible for plant ethylene synthesis, is sensitive to inhibition by a range of 2-oxoacids, competitively with respect to the co-substrate ascorbic acid, and non-competitively with respect to ACC, and to the co-factors, iron (II) and carbon dioxide. The significance of the findings is discussed in terms of the reaction mechanism of ACC oxidase, an enzyme which is related by primary sequence to the family of iron (II)-dependent dioxygenases, most of which use 2-oxoglutarate as a co-substrate. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The enzyme responsible for the final step in the biosynthesis of ethylene in plants, 1-aminocyclopropane-1-carboxylate (ACC) oxidase uses molecular oxygen to catalyse the oxidative breakdown of ACC to ethylene, cyanide and CO_2. It has been shown by primary sequence comparison to be a member of the iron (II)-dependent dioxygenase family of enzymes [1–4]. Most iron (II)-dependent dioxygenases require a 2-oxoacid co-substrate, commonly 2-oxoglutarate, which is oxidized to succinate and CO_2. The two enzymes of this family that apparently do not require a 2-oxoacid co-substrate are isopenicillin *N* synthase (IPNS) [5] and ACC oxidase. IPNS uses no co-substrate other than O_2, whereas ACC oxidase uses ascorbate as a co-substrate [6], apparently oxidizing it stoichiometrically to dehydroascorbate [7]. Also uniquely among the known iron (II)-dependent dioxygenases, ACC oxidase requires CO_2 as an essential cofactor [7–9].

The basis of the CO_2 requirement for ACC oxidase activity is unclear. It may, as in ribulose bisphosphate carboxylase–oxygenase [10] activate the enzyme via a reaction with an amino acid side chain [9], or it may react with one of the substrates, either ACC or ascorbic acid to form a carbamate or carbonate respectively. The concentration of these two secies in solution would be expected to be low, but they may be stabilized by active site interactions: the concentrations of ascorbate and CO_2 required for optimal *in vitro* ACC oxidase activity are higher than are likely to be met *in vivo* [8, 11].

The mechanism and active site of the iron (II)-dependent dioxygenase, prolyl 4-hydroxylase, have been explored by extensive kinetic studies employing structural analogues of 2-oxoglutarate [12], but no comparable work has been done with ACC oxidase.

RESULTS AND DISCUSSION

The enzyme used in the present study was extracted from ripe pears. Elsewhere it has been shown that the pear ACC oxidase studied in crude extracts [13] and purified to electrophoretic homogeneity (Iturriagagoitia-Bueno and John, unpublished) resembles closely ACC oxidase from other fruits. We chose to use an unpurified homogenate because the time-course of ethylene production was linear over the 15 min period of assay permitting kinetic parameters to be determined. The purified enzyme has been reported to be more rapidly inactivated during the reaction period [14]. Figure 1 shows that the pH optimum of the pear enzyme is shifted to a lower pH when non-limiting concentrations of CO_2 are supplied. A similar effect was also shown for ACC oxidase activity from other plants [11, 15].

Figure 2a shows that 2-oxoglutarate clearly inhibits ACC oxidase activity. As 2-oxoglutarate is potentially a chelator of iron (II), and iron (II)-chelators are known to be potent inhibitors of ACC oxidase [6, 16] it was important to determine the extent of iron (II) chelation in its mode of action. Figure 2 shows that, unlike the

†Author to whom correspondence should be addressed.

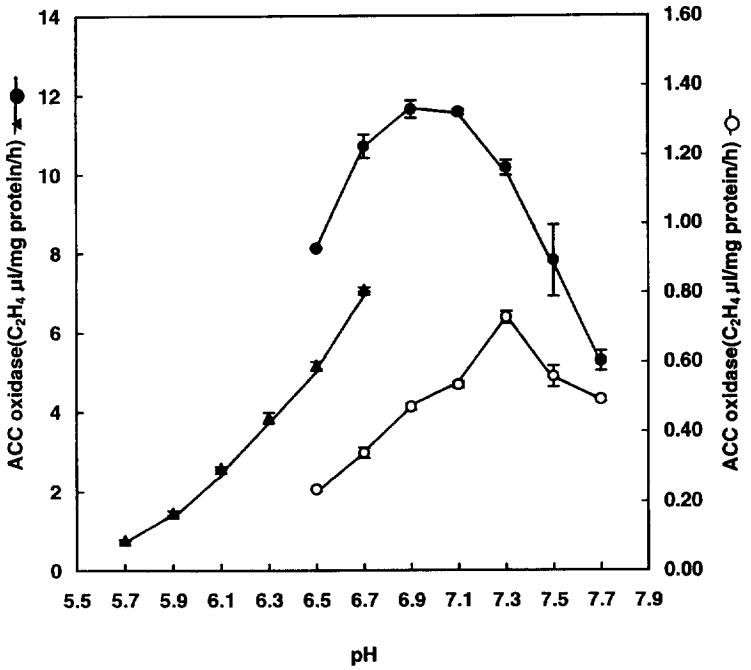

Fig. 1. Effect of pH on ACC oxidase activity in the presence and absence of saturating CO_2 levels. The enzyme assay was carried out in a standard reaction mixture with (▲) 0.2 M MES, (●) 0.2 M MOPS and (○) in 0.2 M MOPS with no addition of CO_2 and bicarbonate.

known iron (II) chelator 1,10-phenanthroline (Fig. 2b), inhibition by 2-oxoglutarate (Fig. 2a) was not enhanced when iron (II) was limiting. Thus we conclude that iron (II) chelation is not a significant factor in the inhibitory effect of 2-oxoglutarate. The effectiveness of 2-oxoglutarate was enhanced when the ascorbate levels were reduced, but inhibition was relatively insensitive to changes in CO_2 levels. Lineweaver–Burk plots (Fig. 3) show that inhibition by 2-oxoglutarate is competitive with respect to ascorbate and non-competitive with respect to CO_2. A similar pattern of kinetics was observed with 3-oxoglutarate (Fig. 4a and b). 2-Oxosuccinate (oxaloacetate) was the most potent inhibitor examined (Fig. 5), and was therefore investigated in more detail. Inhibition was competitive with respect to ascorbate (Fig. 5a) and non-competitive with respect to ACC (Fig. 5b), iron (II) (Fig. 5c) and CO_2 (Fig. 5d). 2-Oxoadipate was less effective, while pyruvate and succinate were ineffective (Table 1).

The observation that 2-oxoacids inhibit competitively with respect to ascorbate, but not with respect to CO_2 or ACC is most simply explained by a binding of the 2-oxoacids at the ascorbate-binding site (presumably ligated at the active site iron (II)), but not at the ACC and CO_2 binding sites. The results also imply that CO_2 and ascorbate do not form a substrate carbonate.

2-oxoadipate is the weakest of the 2-oxoacids tested (Table 1), suggesting that it cannot be accommodated easily by the active site. That 2-oxoglutarate is less effective than both 2-oxosuccinate and 3-oxoglutarate is at first perhaps surprising, since it seems likely that ACC oxidase evolved from a 2-oxoglutarate-using ancestor [3]. However *in vivo*, ACC oxidase is more likely to encounter 2-oxoglutarate, rather than 3-oxoglutarate or 2-oxosuccinate, and may therefore have evolved a structure that binds 2-oxoglutarate less well.

The 2-oxoacids may exist in a number of different equilibrating forms in solution, including cyclic forms; and it is unknown which form binds to the enzyme. Similarly, while ascorbate is apparently stoichiometrically oxidized to dehydroascorbate [7], it cannot be ruled out that it is a ring-opened form of ascorbate that actually reacts at the active site. The inhibition of iron (II)-dependent dioxygenases, such as prolyl 4-hydroxylase, by pyridine dicarboxylates has been taken to imply that 2-oxoglutarate binds in a bidentate fashion to the active site iron (II) [17]. However it is likely that iron(II)-chelators, such as the pyridine dicarboxylates will inhibit simply by iron (II)-chelation provided only that they can be accommodated by the active site. Thus it is only with caution that one can imply a mode of substrate binding from the mode of inhibitor binding.

EXPERIMENTAL

Preparation of experimental material. ACC oxidase was extracted from fully ripe pear fruits (*Pyrus communis* L. cv Conference). Frozen tissue was homogenized in a pestle and mortar with sand and 1 ml mg^{-1} of buffer containing 0.1 M Tricine (pH 7.5), 10% (v/v) glycerol, 30 mM Na ascorbate, 3 mM DTT, 3% (v/w) PVP and 0.1% (v/v) Triton X-100. The homogenate

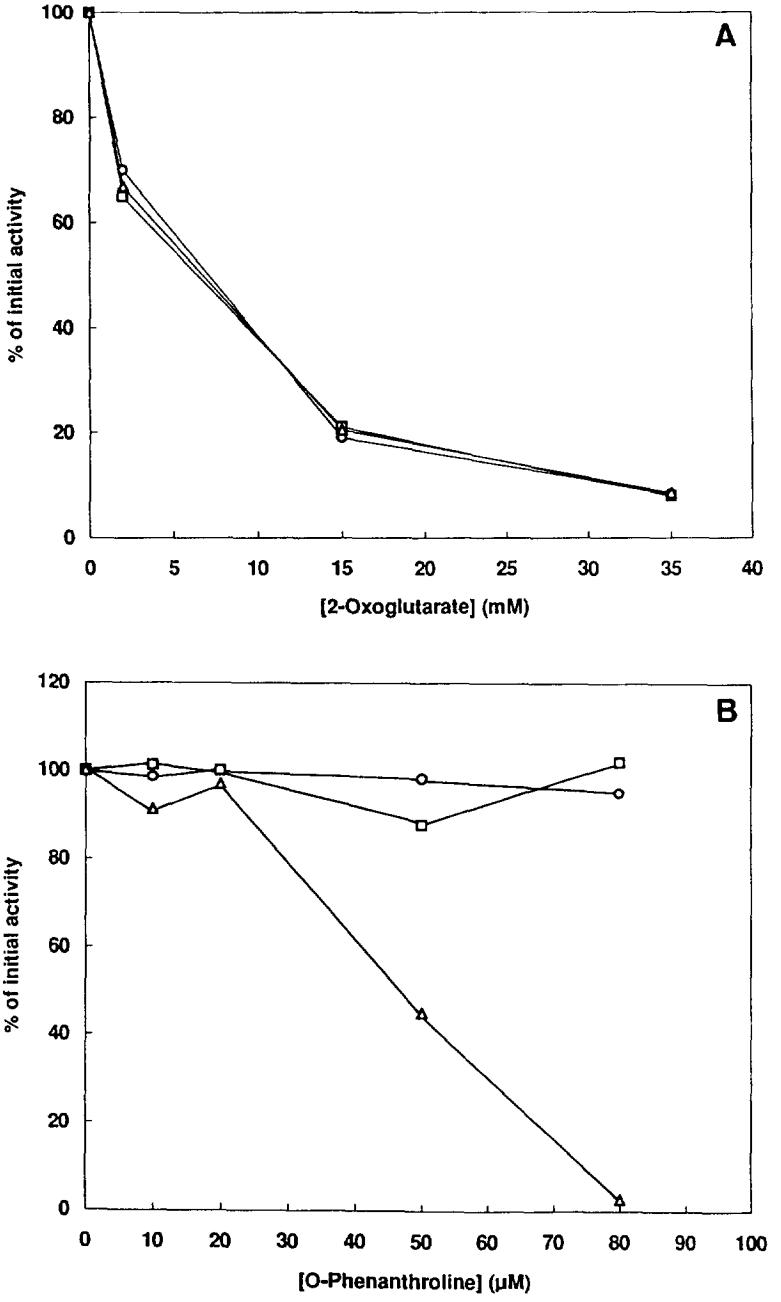

Fig. 2. Effect of iron (II) on the inhibition of ACC oxidase by (a) 2-oxoglutarate and (b) 1,10-phenanthroline. The standard reaction mixture contained ferrous sulphate added at: (○) 150 μM, (□) 100 μM and (△) 20 μM.

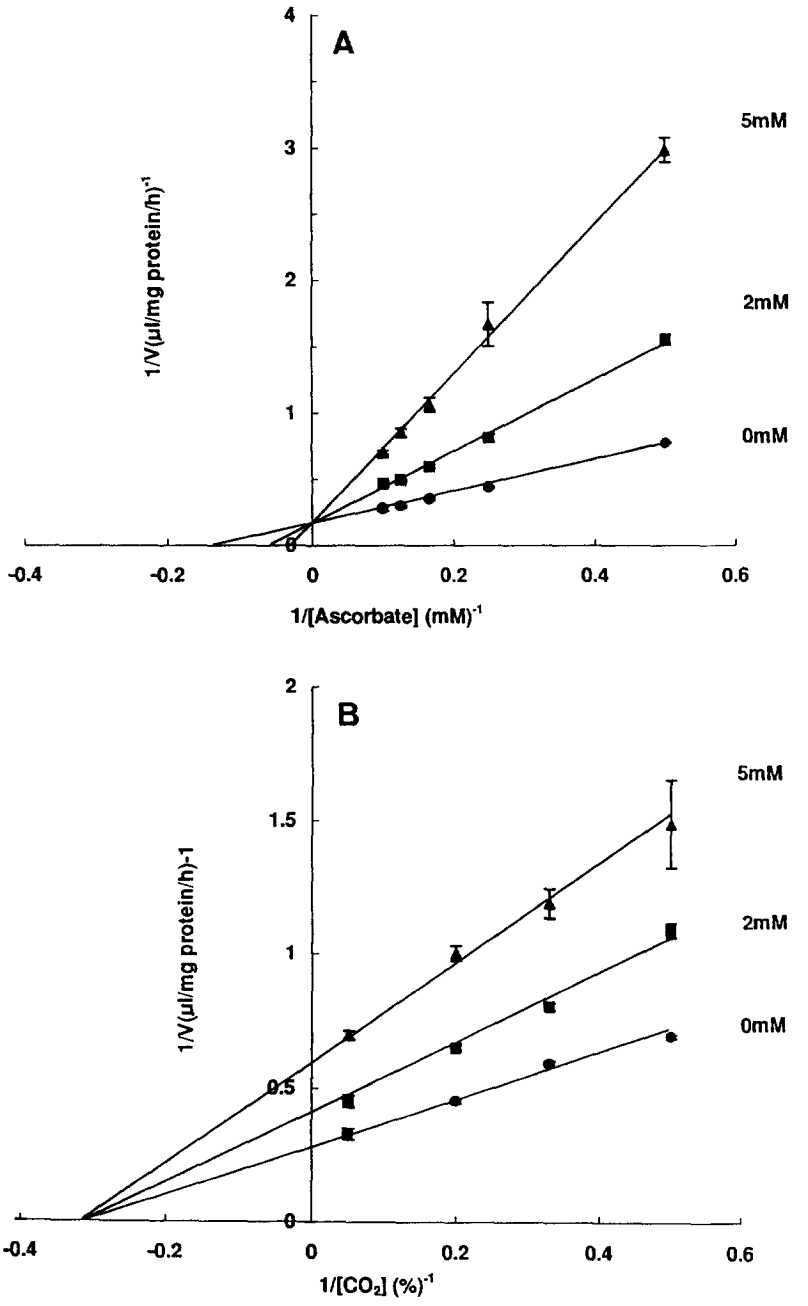

Fig. 3. Lineweaver–Burk plots of the inhibition of ACC oxidase by 2-oxoglutarate with respect to (a) ascorbate and (b) CO_2. In (b) the reaction mixture contained 12 mM sodium ascorbate.

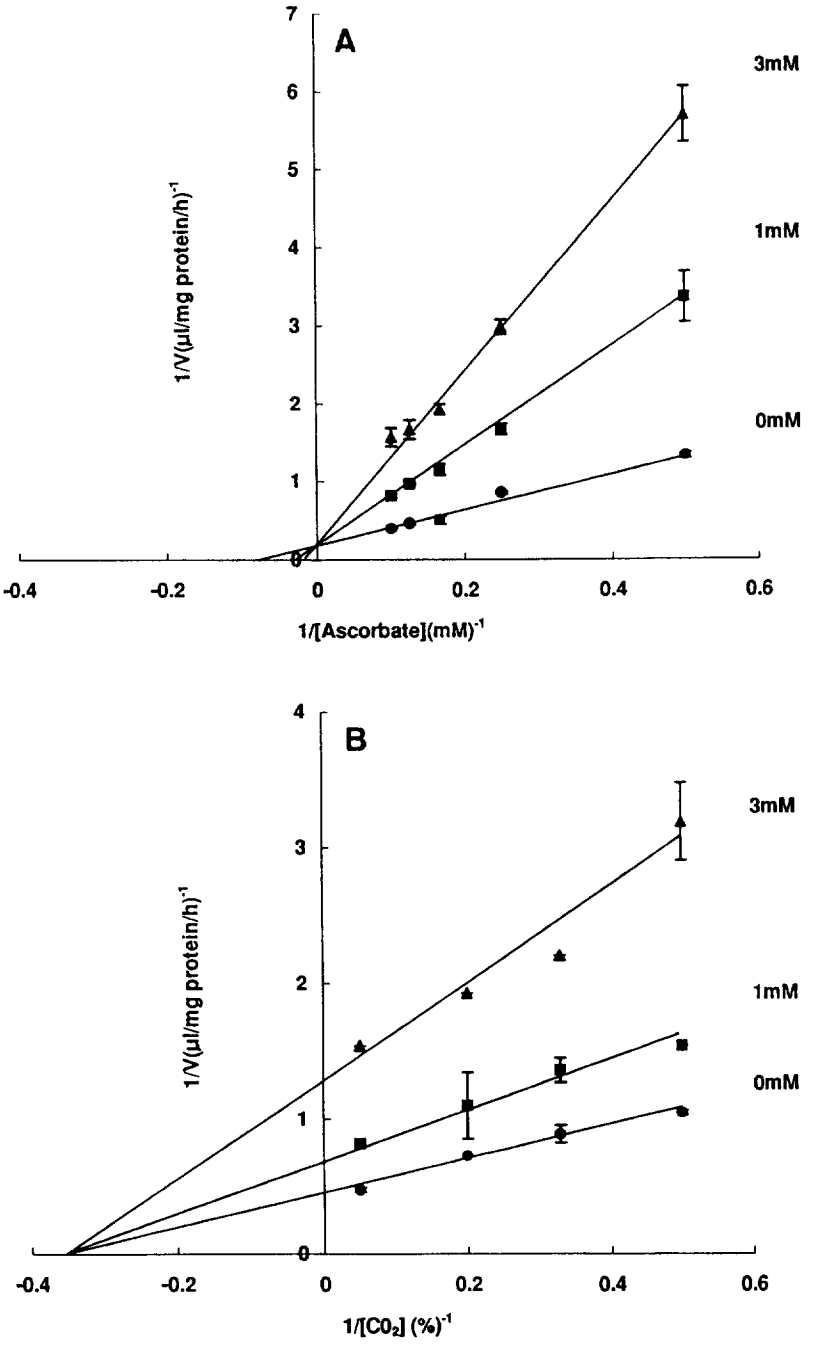

Fig. 4. Lineweaver–Burk plots of the inhibition of ACC oxidase by 3-oxoglutarate with respect to (a) sodium ascorbate and (b) CO_2. In (b) the reaction mixture contained 12 mM sodium ascorbate.

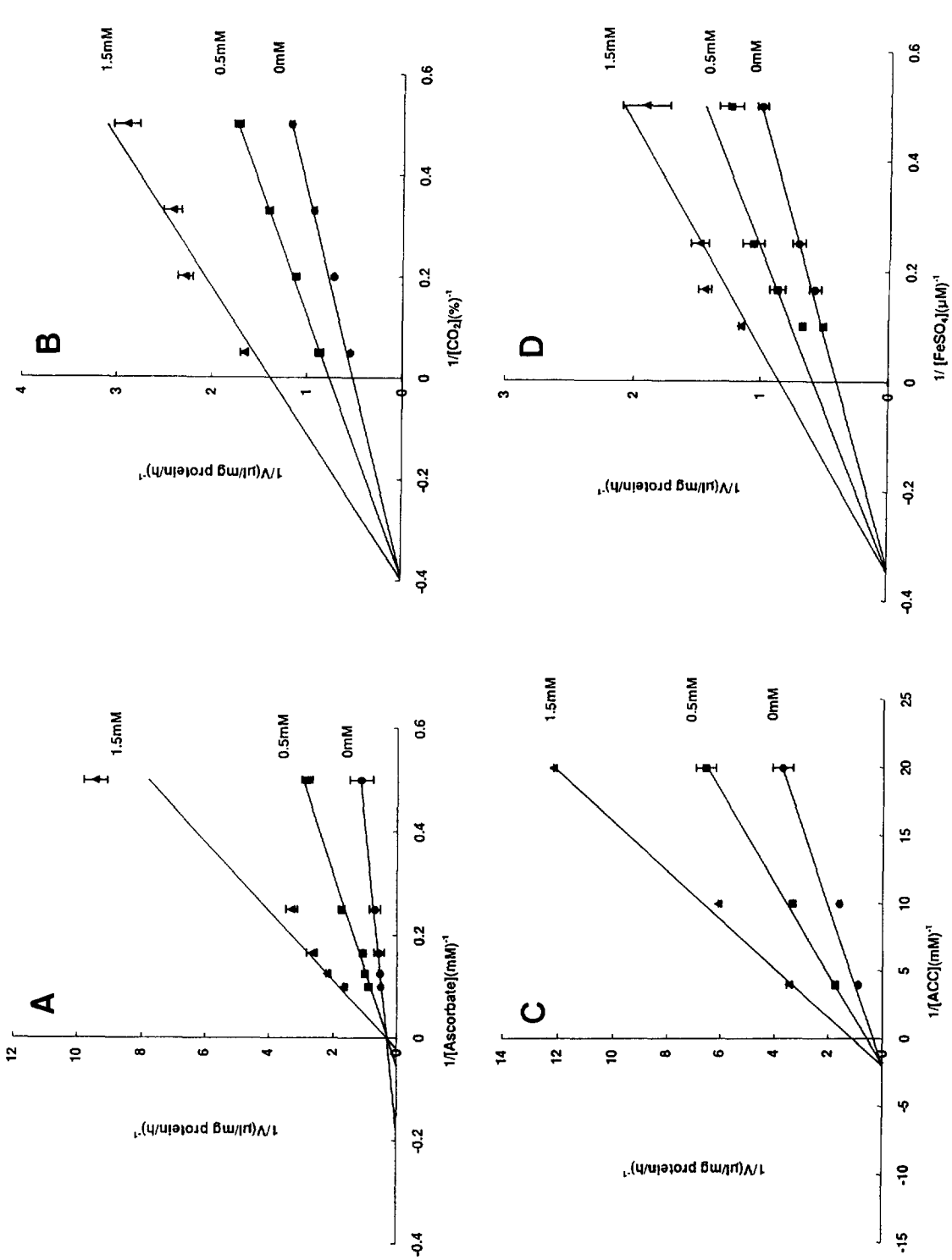

Fig. 5. Lineweaver–Burk plots of the inhibition of ACC oxidase by oxaloacetate with respect to (a) sodium ascorbate, (b) CO_2 (the reaction mixture contained 12 mM sodium ascorbate), (c) ACC and (d) iron (II).

Table 1. Inhibition constants

Compound	K_i (mM)
2-Oxosuccinate	0.24
3-Oxoglutarate	0.86
2-Oxoglutarate	1.35
2-Oxoadipate	≈5
Succinate	>20
Pyruvate	>20

was filtered through 2 layers of muslin and the filtrate centrifuged (20 000 *g*, 20 min). The extract was assayed after passing the supernatant through a Sephadex G-25 Column, equilibrated with 0.1 M Tricine (pH 7.5), 10% (v/v) glycerol, 3 mM DTT.

Enzyme assay. Enzyme activity was determined by measuring the C_2H_4 produced after incubating the enzyme for 15 min at 30° in a standard reaction mixt. Unless otherwise indicated the standard reaction mixt. contained, in a total vol. of 1 ml, 0.2 M MOPS (pH 6.9), 10% (v/v) glycerol, 30 mM Na ascorbate, 0.1 mM $FeSO_4$ and 21 mM $NaHCO_3$. The atmosphere above the reaction medium was enriched with 20% CO_2. Whenever CO_2 levels were varied the HCO_3^- concn in the reaction medium was also varied, so that it was in equilibrium with the CO_2 concn provided, as calcd by the method of ref. [18]. The pH of the reaction medium was checked after the enyme assay. The use of both $NaHCO_3$ and CO_2 avoided significant shifts in reaction medium pH due to H^+ from the ionization of carbonic acid. All determinations were made in triplicate and the results are expressed as means±s.e. Values of K_i were calculated using an Excel curve-fitting program, and data from experiments in which the concns of ascorbate and inhibitor were varied.

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