Phytochemistry, 1980, Vol. 19, pp. 1931–1933. © Pergamon Press Ltd. Printed in England. 0031–9422/80/0901–1931 $02.00/0

REACTIVATION BY COPPER OF PHENOLASE PRE-INACTIVATED BY OXALATE

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(*Revised received* 4 *January* 1980)

Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; phenolase; enzyme inhibition mechanism; inhibitor; ammonium oxalate; enzyme reactivation; copper; chloroplasts.

Abstract—The activity of spinach chloroplast phenolase which had been repressed by ammonium oxalate was restored by adding copper. Oxalate appears to bind to the enzyme at a single site, the binding paralleling the inhibition produced at neutral pH. The inhibition of oxalate is due to its binding with copper at the active centre to form an inactive complex, the oxalate moiety of which is releasable when more copper is added. Similar reactivation by copper was obtained with pure mushroom phenolase.

INTRODUCTION

Ammonium oxalate is a natural inhibitor of spinach phenolase present in the chloroplasts [1, 2]. With oxalate of one order lower concentration than that found *in vivo*, complete inhibitions were achieved at acidic pH (e.g. by 1 and 5 mM oxalate at pH 5.0 and 5.5, respectively), and even at neutral pH, pre-incubation of the enzyme with oxalate could cause the total loss of the activity. 50% inhibition was also induced by freezing with a lower concentration (0.25 mM) of oxalate. Purified mushroom phenolase was similarly inhibited [2]. The common inhibition mechanism has remained unsolved, and its elucidation is described in the present paper.

RESULTS AND DISCUSSION

Reactivation of the enzyme by copper

In a previous paper [2], conditions were described (left column, Table 1) which completely inactivate the spinach chloroplast phenolase in the presence of oxalates. Since the enzyme contains copper as a functional group [3, 4] and inactivation by organic acids has been correlated to their interaction with the metal [5–7], the effect of copper on the inactivated enzyme was examined, and it was found that it completely recovered its activity without any prolonged pre-incubation (Table 1). Fig. 1 illustrates the relationship between the copper concentration and reactivation. Fairly low concentrations of copper were effective. Activation was

Table 1. Reactivation by cupric ion of spinach phenolase pre-inactivated by treatments with ammonium oxalate

	Inactivation conditions*		Activity after adding copper (M)	
	pH	Oxalate concn (mM)	10^{-5}	10^{-4}
Without pre-incubation	5.0	1	20	63
	5.5	5	64	103
	6.0	10	58	110
With pre-incubation				
18 hr at 0°	6.8	20	82	101
18 hr at −20°	6.8	2	67	90

* Based upon the data in refs. [1 and 2]. The 0.5 ml enzyme–oxalate mixtures contained 0.1 ml enzyme, 0.2 ml 0.2 M Na-Pi buffer or citrate–phosphate buffer and 0.2 ml known concentrations of ammonium oxalate. To the mixtures were added successively 2 ml the same buffer, 1 ml 10 mM sulphanilic acid and 1 ml known concentrations of cupric nitrate, and the activity was immediately estimated by adding 0.5 ml 10 mM catechol, which is expressed as per cent of that of the controls run under the same experimental conditions without oxalate. No activity could be detected in the pre-inactivated enzyme when measured without copper.

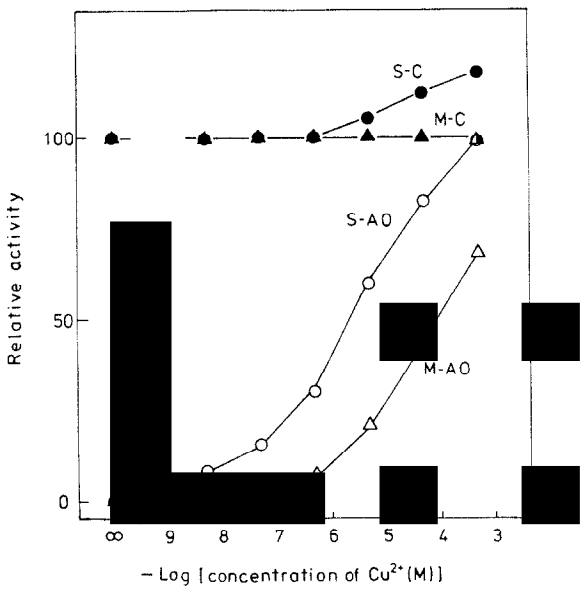

Fig. 1. Relationship between the copper concentration and reactivation of the inactivated spinach and mushroom phenolases. Copper was given as cupric nitrate, and the enzyme pre-inactivated with 5 mM ammonium oxalate (AO) at pH 5.5 (see the Table 1 legend). Activities are expressed as per cent of that of the enzyme without AO-treatment (controls) and measured without copper. S–AO and S–C represent the enzymes pre-treated with AO and the controls, respectively, for spinach, and M–AO and M–C, for mushroom.

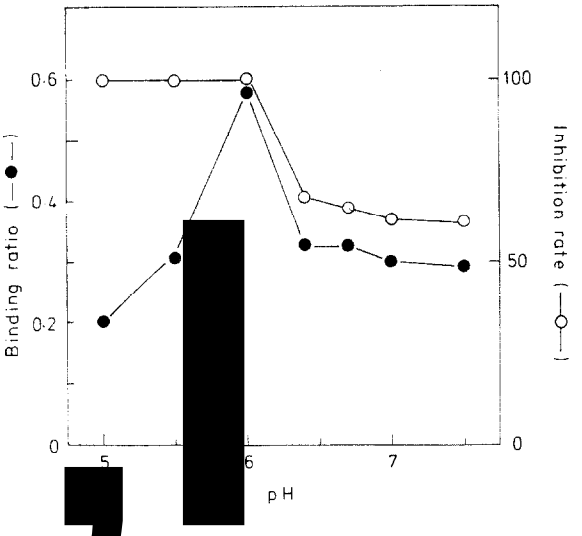

Fig. 2. Relationship at varied pH of the oxalate binding to the inhibition rate in spinach enzyme after 18 hr dialysis with 5 mM ammonium oxalate. The radioactivities in the dialysis bag (A) and those in the medium (B) were separately determined and the binding ratios are expressed as (A − B)/B. The enzyme activities were measured after the dialysis with oxalate (a) and without it (b). and the inhibition rates are given as (b − a)/b × 100.

also achieved with the inactivated mushroom phenolase, although with less efficiency. Reactivation was independent of the cupric salt applied.

Effect of removing oxalate from the inactivated enzyme

We then determined whether the enzyme activity could be recovered when oxalate is removed from the enzyme solution whose activity has been completely inhibited by treatments described in Table 1. No restoration in activity was observed in the enzyme which had been pre-incubated with oxalate for a long time, although 25–35% recovery was achieved in the enzyme from which oxalate was removed without pre-incubation (see Experimental). It thus seems that the inhibition was either caused by masking the active centre with oxalate or by the release of copper with it.

Binding of oxalate to the enzyme

It was then observed that oxalate was bound to the protein of spinach chloroplasts. Using radioactive ammonium oxalate as ligand, the binding ratio was determined at various pHs by equilibrium dialysis (Fig. 2). The ratio was almost parallel to the inhibition at neutral pH, being maximal at pH 6.0 where the inhibition was complete, although it became rather lower at acidic pH where no enzyme activity was detectable. Essentially identical results were obtained with mushroom tyrosinase. Concentration dependency of oxalate binding was analysed by Scatchard plot [8], which gave a straight line, showing the presence of one type of binding site, i.e. the binding occurred at the active centre.

The enzyme–oxalate mixtures were subjected to elution from Sephadex column and to electrophoresis on cellogel plate, after being pre-equilibrated with equal concentrations of non-radioactive ammonium oxalate. No other radioactivity could be detected than that of ammonium oxalate, showing that the bound oxalate is released under the above experimental conditions.

From these data, it is concluded that phenolase inhibition by oxalate is induced by its binding with the functional copper at the active centre of the enzyme to form an inactive enzyme–oxalate complex and that the oxalate moiety of this complex can be released, probably as a chelate with copper [6]. Formation of such a complex may take part in the latency of phenolase of spinach chloroplasts [9].

EXPERIMENTAL

Enzyme solns. Enzyme soln of spinach was prepared from its chloroplast Me_2CO power according to ref. [9]. It was dialysed against 10 mM Na–Pi buffer pH 6.8 to remove a small amount of ammonium oxalate [2]. The dialysate contained 3.0 mg protein in 1 ml. Mushroom tyrosine (phenolase) (Sigma Grade III) was dissolved in the same buffer to obtain an enzyme soln containing 0.20 mg protein in 1 ml.

Removal of oxalate from the enzyme. This was carried out with Sephadex G-25 column (2.5 × 30 cm) which had been equilibrated with the same buffer. One ml of the enzyme–oxalate mixtures containing 0.2 ml enzyme, 0.4 ml 0.2 M Na-Pi buffer at the desired pH (0.1 M citrate–0.2 M Na_2HPO_4 for pH 5.0) and 0.4 ml of the known concns of oxalate was applied within 3 min after mixing or following the pre-incubation and the enzyme activity was estimated for a definite vol. of the effluent at void vol. Controls without oxalate were similarly treated followed by estimation of the activity.

Measurement of enzyme activity. This was done colorimetrically, as already described [2].

Oxalate binding experiments. This was carried out by ordinary equilibrium dialysis. Enzyme soln (0.5 ml) was placed in a dialysis bag (Visking cellulose tubing 18/32), which was immersed in 10 ml of medium containing 400 mol of Na-Pi buffer at appropriate pH (citrate–phosphate buffer was used for pH 5.0) and ammonium oxalate (AO) (in the experiment in Fig. 1, 1 μmol of radioactive plus 49 μmol of non-radiactive AO were added; in another experiment, where AO concn was varied, one fiftieth was given as radioactive salt). Dialysis was run for 18 hr at 0° without stirring. A preliminary experiment showed that an equilibrium was attained within 4–5 hr dialysis under the above conditions. After dialysis, an aliquot (0.1 ml) was withdrawn and quantified for its radioactivity using Beckman LS 245 scintillation spectrophotometer. The errors were less than 1.5%.

Radioactive ammonium oxalate (AO). This was prepared from [U-^{14}C] calcium oxalate (Radiochemical Centre, Amersham). To the ampoule containing 50 μCi of the salt (74 mCi/mmol), 7.21 mg of non-labelled $(COO)_2Ca \cdot H_2O$ were added with 1 ml 5 N HCl and the mixture stood for 2 days. The content was carefully transferred to a test-tube and the free acid was thoroughly extracted with several changes of EtOAc, and the combined extracts were evapd *in vacuo* at 35° until a small vol. of H_2O remained. Five ml conc NH_3 were then added, and the soln re-evapd (by this procedure, Cl^- could be removed as NH_4Cl). The final residue of AO was dissolved in 5 ml H_2O, and thus 10 mM of radioactive AO soln was obtained, whose radioactivity was 6.41 μCi per 1 ml and 0.641 μCi per μmol. It was confirmed that the soln contained only AO as radioactive substance on a radiochromatogram, after development with *n*-BuOH–EtOH–18% NH_3 (4:1:5) as irrigant.

Scatchard analysis. A Scatchard plot was made at pH 6.8 of the oxalate binding by spinach enzyme, bound oxalate being expressed as molar number (nmol) detected in 0.1 ml of 10.5 ml in the total dialysis system. The binding constant, obtained from the negative reciprocal of the slope, was found to be 1.4×10^{-4} M.

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