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PROPERTIES OF NADP-MALIC ENZYME FROM GLUMES OF DEVELOPING WHEAT GRAINS

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Key Word Index—*Triticum aestivum*; Gramineae; wheat glumes; NADP malic enzyme; properties.

Abstract—Properties of partially purified NADP-malic enzyme (EC 1.1.1.40) from glumes of developing wheat grains were examined. The pH optimum for enzyme activity was influenced by malate and shifted from 7.3 to 7.6 when the concentration of malate was increased from 2 to 10 mM. The K_m values, at pH 7.3, for various substrates were: malate, 0.76 mM; NADP, 20 μM and Mn^{2+}, 0.06 mM. The requirement of Mn^{2+} cation for enzyme activity could be partially replaced by Mg^{2+} or Co^{2+}. Mn^{2+} dependent enzyme activity was inhibited by Pb^{2+}, Ni^{2+}, Hg^{2+}, Zn^{2+}, Cd^{2+}, Al^{3+} and Fe^{3+}. During the reaction, substrate molecules (malate and NADP) reacted with enzyme sequentially. Activity of malic enzyme was inhibited by products of the reaction viz pyruvate, HCO_3^- and $NADPH_2$. At a limiting fixed concentration of NADP, these products induced a positive cooperative response to increasing concentrations of malate.

INTRODUCTION

Photosynthetic activity of leaves, stem and reproductive structures collectively contributes towards yield in crop plants [1]. However, the contribution made by each part varies with the crop species. In cereals, net photosynthetic activity in the reproductive organs is relatively high and can contribute as much as 50–75% of the photosynthate to the grain [2–4]. Recent studies conducted in wheat have indicated that all the parts of a spikelet such as awn, glume lemma and palea contain chlorophyll and are capable of photosynthetic CO_2 fixation [5, 6]. These studies have further revealed that in these parts, PEP carboxylase is more active than RuBP carboxylase, which may be re-fixing dark respired or photorespired CO_2. Recent experiments conducted in our laboratory in the presence of $^{14}CO_2$ have shown that the first product of CO_2 fixation in these tissues is oxaloacetate and malate [Ram and Singh, unpublished], rather than 3-PGA as is the case in the C_3 pathway, confirming that the tissues surrounding grain have the capacity for C_4 metabolism. NADP-malic enzyme plays a significant role in C_4 metabolism in the malate decarboxylation, liberating CO_2, which again is refixed through the Calvin cycle. Though the properties of this enzyme are well studied from C_4 [7–12] and CAM [13, 14] plants, it is yet to be characterized from reproductive parts. Here, we report some of the properties of NADP-malic enzyme isolated from glumes of wheat ear.

RESULTS AND DISCUSSION

Purification of the enzyme

NADP-malic enzyme from glumes of developing wheat grains was purified by a procedure involving ammonium sulphate fractionation, DEAE-cellulose chromatography and gel filtration through Sephadex G-200. Specific activity of the final preparation was about 6-fold higher (554 nmol of NADP reduced/mg protein/min) than that of the crude homogenate, though its protein content decreased by a factor of about 120-fold. Efforts to further increase specific activity of the purified preparation were unsuccessful due to the instability of the enzyme during purification. Attempts to stabilize it by altering the composition of extraction and elution buffers, by supplementation with Mn^{2+} or Mg^{2+}, by varying the concentration of cysteine or β-mercaptoethanol or including 10–20% (v/v) glycerol had no beneficial effect. All the subsequent experiments were conducted with this partially purified enzyme preparation which was completely free of NADP-malate dehydrogenase, NADP(H) oxidase and NADPH lactate dehydrogenase activities.

Stability

The enzyme from wheat glumes was rather unstable. Enzyme activity in both crude and purified enzyme preparation decreased by about 30% in 24 hr during storage at 5°. Incubation of purified enzyme at 40° for 15 min resulted in a complete loss of activity and a 14% decrease occurred when the incubation was carried at 30°. In contrast to NADP-malic enzyme from wheat glumes, the activity of the enzyme from *Bryophyllum* was not affected by preincubation at 55° up to 30 min. In fact, in the case of *Bryophyllum*, the optimum temperature for activity was 50° [13].

Molecular weight

The enzyme was eluted as a single peak on gel filtration with Sephadex G-200. Its estimated M_r was 255 000 which is within the reported range of 227 000–258 000 from other plant species [12, 13, 15].

Effect of substrates

A normal hyperbolic response was obtained on increasing concentrations of either malate or NADP in an otherwise standard assay mixture. The K_m values for

malate and NADP were 0.76 mM and 20 μM, respectively. The K_m for NADP is in accord with that reported for the enzyme from several other plants [7, 9, 12, 16, 17]. A similar K_m value of 0.77 mM for malate for NADP-malic enzyme was reported for the enzyme from wheat germ [18]. In contrast to the malate producing C_4 plants, this comparatively high K_m value for malate is characteristic of the enzyme from C_3 and CAM plants [10].

pH optimum

In agreement with observations of earlier workers [7, 13, 15, 19], the pH optimum for enzyme activity was influenced by malate concentration. In an otherwise standard assay mixture, increasing the concentration of malate from 2 to 10 mM shifted the pH optimum for the enzyme from 7.3 to 7.6. The pH response of the enzyme from wheat glumes is like that of the enzyme from C_3 and CAM plants rather than that from malate producing C_4 plants [10, 17].

Effect of metal ions

The partially purified enzyme exhibited an absolute dependence on the presence of a divalent cation either Mn^{2+} or Mg^{2+} for its activity. The K_m values for these cations were 0.06 and 1.1 mM, respectively. At their saturating concentrations, Mg^{2+} or Co^{2+} gave 60 and 30% of the activity obtained with Mn^{2+}. In the absence of Mn^{2+}, other metal ions like Na^+, NH_4^+, K^+, Ni^{2+}, Zn^{2+}, Ba^{2+}, Ca^{2+}, Sr^{3+} and Al^{3+}, in the concentration range of 1–10 mM, were totally ineffective. A similar order of effectiveness of metal ions for activity of this enzyme has been reported in mango [16], grapes [20] and *Bryophyllum* [13]. However, the enzyme from maize [7] and potato tubers [21] was more active with Mg^{2+} than with Mn^{2+}.

The Mn^{2+} dependent activity of malic enzyme was completely inhibited by 5 mM Hg^{2+}, Pb^{2+}, Zn^{2+}, Cd^{2+} or Fe^{3+}. However, Co^{2+}, Al^{3+} and Ni^{2+} at this concentration inhibited the activity by 72, 43 and 53% respectively. Na^+, NH_4^+, K^+, Ba^{2+}, Ca^{2+} and Sr^{3+} had no effect. The requirement of sulphydryl groups for functioning of this enzyme has been deduced earlier [11–13] and some of these heavy metals presumably interfere with the enzyme activity due to their reactivity with sulphydryl groups. Among the various anions examined, S^- and to a lesser extent SO_3^{2-} were inhibitory. Cl^-, Br^-, F^-, I^- and SO_4^{2-} had no effect. Inhibition by SO_3^{2-} has been earlier shown to be competitive with Mn^{2+} and NADP for the enzyme from maize leaves [9].

Effect of organic acids

Under standard conditions of assay, oxalate was a potent inhibitor and at 2.5 mM it completely suppressed the enzyme activity. This suppression of activity was not due to chelation, as even at higher concentrations of Mn^{2+} (5 and 10 mM), the inhibitory effect was not reversed. α-Ketoglutarate and oxaloacetate at 5 mM, diminished the enzyme activity by 41 and 72%, respectively, whereas succinate showed no effect. From double reciprocal plots, the inhibition by α-ketoglutarate and oxaloacetate was characterized as uncompetitive and competitive respectively, against malate. The uncompetitive inhibition by α-ketoglutarate has also been observed for enzyme from maize leaves [12]. However, the observed competitive inhibition by oxaloacetate is at variance with results of Asami *et al.* [12] but is in accord with those of Bhagwat *et al.* [11]. Inhibition by oxalate was also reported for malic enzyme from maize leaves by the latter workers [11].

Mechanism of reaction

In order to obtain information whether the enzyme catalyses the reaction through a sequential or ping pong mechanism, its activity was determined at three fixed concentrations of NADP at varying concentrations of malate (Fig. 1A) and vice versa (Fig. 1B). In both the experiments, the family of curves intersected at a point between two ordinates thus suggesting a sequential rather than ping pong binding and release of substrates and products, respectively. These results are in conformity with the earlier results for NADP-malic enzyme from *Pennisetum purpureum* [8]. Further experiments to ascertain whether the addition of the substrates was ordered or random were confounded by rather complex kinetic responses in presence of products, namely, NADPH, pyruvate and HCO_3^- particularly at fixed sub-saturating concentrations of NADP (see below).

Effects of products

Malic enzyme catalyses the reversible oxidative decarboxylation of malate with products of the forward reaction being pyruvate, HCO_3^- and NADPH. Results in Fig. 2A show that pyruvate acted as an uncompetitive inhibitor against malate. In this experiment, where a saturating amount of NADP was used, a maximum inhibition of 20% was recorded. Inhibition by pyruvate was, however, more marked at subsaturating levels of NADP. Thus at 25 μM NADP, 5 mM pyruvate decreased enzyme activity by more than 50% and this inhibitory effect was partially relieved at higher concentrations of NADP. The Lineweaver–Burk plot indicated a mixed type of inhibition of pyruvate against NADP (Fig. 2B). Unlike the uncompetitive inhibition by pyruvate against malate (see Fig. 2A), different results were obtained when its effect was studied at a fixed sub-saturating concentration of NADP with malate as a variable substrate (Fig. 3A). The inhibition was maximum at lower levels of malate but was progressively reversed by increasing concentrations of malate. In a double reciprocal plot of the results, a normal linear curve was obtained in control tubes containing varying concentrations of malate. However, in the presence of pyruvate, the curves obtained were concave upwards (data not presented) thus denoting that pyruvate elicited a positive cooperative effect by malate on the functioning of the enzyme. Possibly, under these conditions, interaction of a few initial molecules of the substrate is essential to convert the enzyme to its functionally efficient form. When a sub-saturating concentration of malate was used and the concentration of NADP varied (Fig. 3B), the inhibition was not reversed by NADP which again is in contrast to conditions of saturating levels of malate.

Effect of bicarbonate

Inhibition of enzyme activity by HCO_3^- in the presence of varying concentrations of malate and either saturating

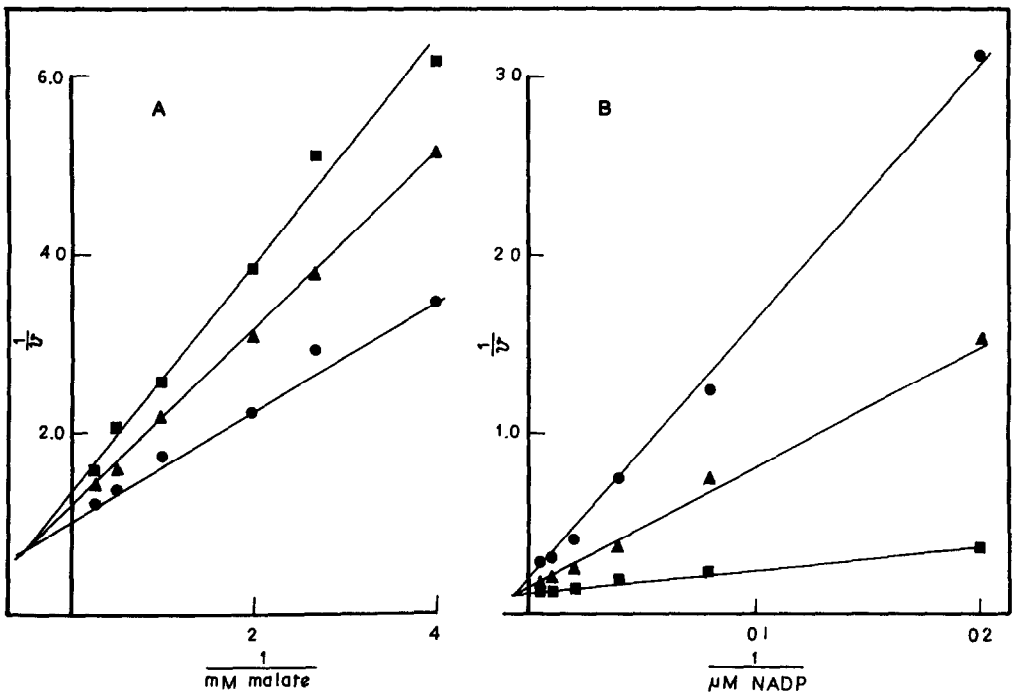

Fig. 1. (A) Effect of varying concns of malate on enzyme activity at three fixed concns of NADP. The enzyme was assayed spectrophotometrically as described in the Experimental, except that the concn of malate and NADP in an otherwise standard assay mixture were varied as shown in the figure. Standard reaction mixture in a final volume of 2 ml, contained: Tris–HCl (pH 7.3), 150 μmol; malate, 10 μmol; NADP, 0.4 μmol; Mn^{2+}, 2 μmol and 0.2 ml of partially purified enzyme. Three different concns of NADP were: ■, 50 μM; ▲, 100 μM; ●, 200 μM. $\frac{1}{v}$ denotes reciprocal of nmol of NADP reduced per min in the reaction mixture. (B) Effect of varying concn of NADP at three fixed concns of malate. Experimental details were as described in Fig. 1A except that enzyme activity was determined at three concns of malate. ●, 0.5 mM; ▲, 1.0 mM; ■, 5.0 mM with varying concn of NADP as shown in the figure.

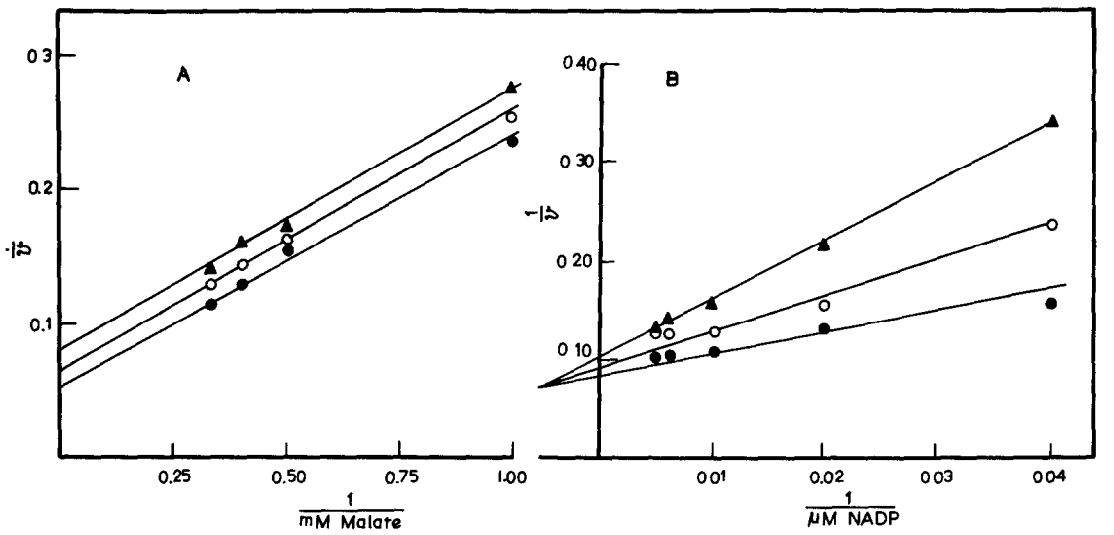

Fig. 2. Double reciprocal plots of effect of pyruvate on enzyme activity at varying concns of malate (A) or NADP (B). Activity of NADP-malic enzyme was determined in the presence of pyruvate in an otherwise standard reaction mixture (see Experimental) except that the concn of malate (Fig. 2A) or NADP (Fig. 2B) was varied as indicated. Concns of pyruvate used were: ●, 0 (control); ○, 2.5 mM; ▲, 5 mM. $\frac{1}{v}$ represents reciprocal of nmol of NADP reduced per min in the reaction mixture.

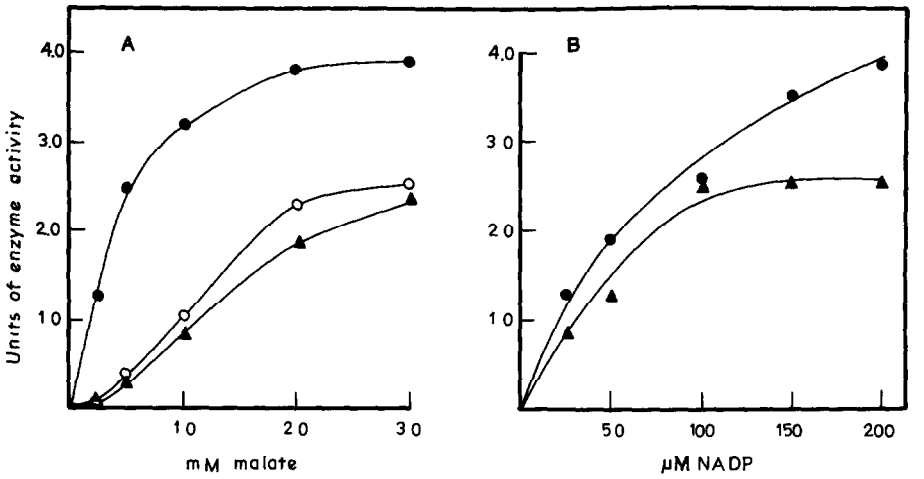

Fig. 3. (A) Effect of pyruvate on enzyme activity at varying concns of malate and a fixed sub-saturating concn of NADP. Activity of the enzyme was determined in the presence of pyruvate at the indicated concn of malate and 0·05 μmol of NADP. Concn of the other components was the same as in the standard assay mixture (see Experimental). Concns of pyruvate used were: ●, 0 (control); ○, 2.5 mM; ▲, 5.0 mM. (B) Effect of pyruvate on enzyme activity at varying concn of NADP and a fixed sub-saturating concn of malate. Experimental details were as described in Fig. 3A except that the concn of malate in the reaction mixture was 1 μmol and that of NADP was varied as shown.

or sub-saturating fixed concentration of NADP is shown in Figs 4 and 5, respectively. At saturating amounts of NADP and with 1 mM malate, 5 mM bicarbonate reduced activity by 45% and this inhibition was progressively reversed with increasing concentration of malate and was apparently competitive. These results are in agreement with the earlier report [19] that HCO_3^- inhibited the enzyme activity by lowering affinity for malate, though by binding at a different site. Similarly, inhibition of light-dependent, 3-phosphoglycerate-stimulated decarboxylation of malate by bicarbonate in bundle sheath chloroplasts of C_4 plants has been shown and this effect was also overcome by higher concentrations of malate [22]. As shown in Fig. 5, at sub-saturating levels of NADP, bicarbonate evoked a sigmoidal response to increasing concentrations of malate and the maximum activity obtained was significantly lower than that in the control. The $S_{0.5}$ values for malate at their respective V_{max}, increased from 0.4 mM to 1.3 mM in the presence of 5 mM HCO_3^-. This again suggests that in the presence of HCO_3^-, malate acts as a positive effector of the enzyme.

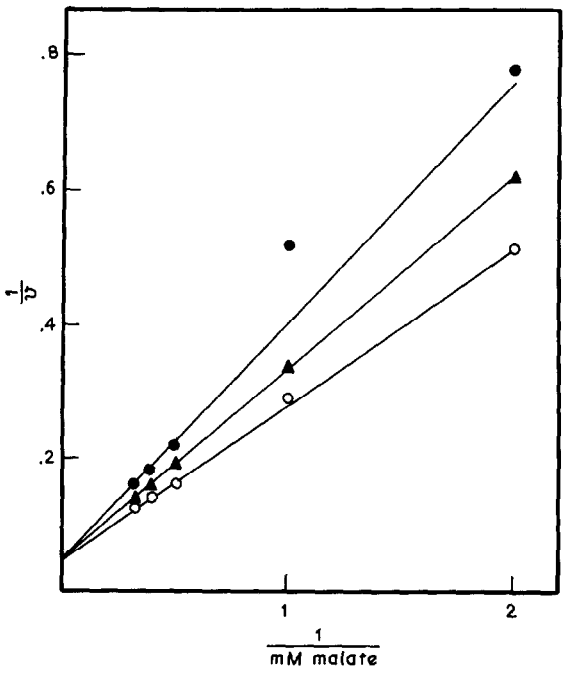

Fig. 4. Effect of HCO^-_3 at varying concns of malate on enzyme activity. Experimental details were as described in Fig. 1A except that the reaction mixture contained sodium bicarbonate at the following concns: ○, 0 (control); ▲, 2.5 mM; ●, 5.0 mM. $\frac{1}{v}$ denotes reciprocal of nmol of NADP reduced/min in the reaction mixture.

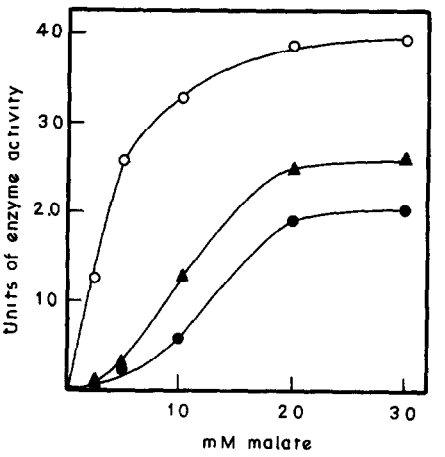

Fig. 5. Effect of HCO^-_3 at varying concns of malate at a fixed sub-saturating concn of NADP. Details of the experiment were as in Fig. 3A except that pyruvate in the reaction mixture was replaced with sodium bicarbonate at the following concns: ○, 0 (control); ▲, 2.5 mM; ●, 5.0 mM

Effect of NADPH

Under conditions of standard assay (NADP saturation), inhibition of NADPH was reversed by increasing concentrations of malate. Thus a 33% inhibition caused by 0.05 mM NADPH at 0.5 mM of malate was diminished to 10% at 3 mM of malate. In the presence of NADPH, a sigmoidal curve with varying malate concentration was observed, implying a positive cooperative interaction with the enzyme molecule (data not shown). At saturating malate and varying NADP concentrations, NADPH caused a mixed type of inhibition (Fig. 6). Coombs *et al.* [8] had, however, reported that NADPH inhibits the activity non-competitively with respect to NADP. At sub-saturating NADP, NADPH induced a positive cooperative response by malate and inhibition was partially reversed by high concentrations of malate (Fig. 7). At lower concentration of malate, NADPH completely abolished enzyme activity. At sub-saturating malate concentration and varying NADP levels (Fig. 8) the extent of inhibition remained more or less constant. However, the double reciprocal plot of the data (not shown) in the presence of NADPH, instead of being linear, gave convex shaped curves indicating that NADPH induces a negative cooperative response of the enzyme to NADP. Thus at sub-saturating levels of malate, the presence of NADPH will restrain the expected enhancement in activity with increasing concentrations of NADP due to conversion of the enzyme to a form less responsive to increasing concentrations of the substrate. The possibility of regulation of NADP-malic enzyme activity by NADPH/NADP ratio has also been suggested earlier [12].

The precise role of NADP-malic enzyme in reproductive parts of developing cereal grains is not yet fully understood. Properties of the enzyme from wheat glumes largely resemble those from leaves of C_3 plants. In these plants, this enzyme is assumed to be important in the generation of NADPH for anabolic reactions [23, 24]. The observed effects of products of the reaction namely pyruvate, bicarbonate and NADPH, exhibit interesting features which could be relevant for *in vivo* regulation of the enzyme activity. Thus whenever the intracellular concentrations of NADP and/or malate are limiting, their excessive utilization in this reaction will be curtailed if the products of the reaction are not consumed rapidly. Such an effect of the products would consequently facilitate diversion of limited supply of these metabolites for other pathways. This constraint, by high intracellular levels of the products, will partly be overcome when the concentration of malate approaches near saturating levels for enzyme activity due to the product-induced cooperative interaction of malate with the enzyme (Figs 3, 5 and 7). Under these conditions, functioning of the enzyme will

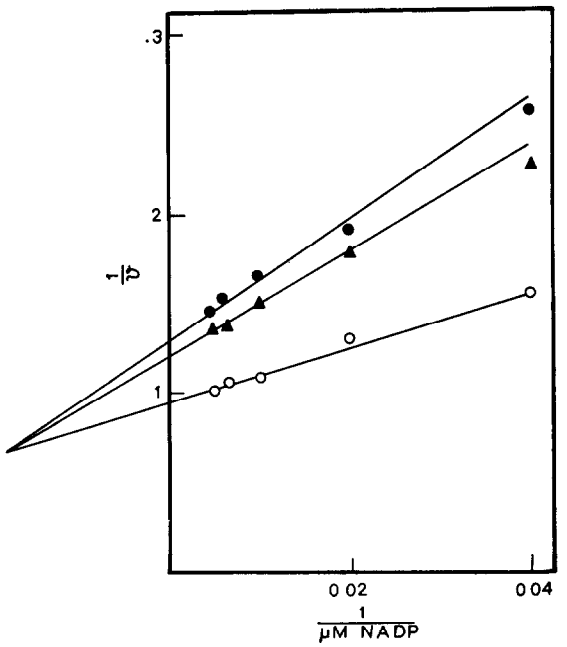

Fig 6. Effect of NADPH at varying concns of NADP on enzyme activity. Experimental details were as in Fig. 1B except that the reaction mixture contained the following concns of NADPH: ○, 0 (control); ▲, 0.05 mM; ●, 0.1 mM.

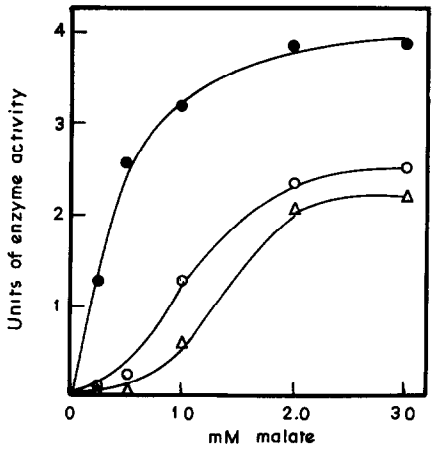

Fig. 7. Effect of NADPH on enzyme activity at varying concns of malate and a fixed sub-saturating concn of NADP. Details of the experiment as in Fig. 3A except that instead of pyruvate, the reaction mixture contained NADPH at the following concns: ●, 0 (control); ○, 0.05 mM; △, 0.1 mM.

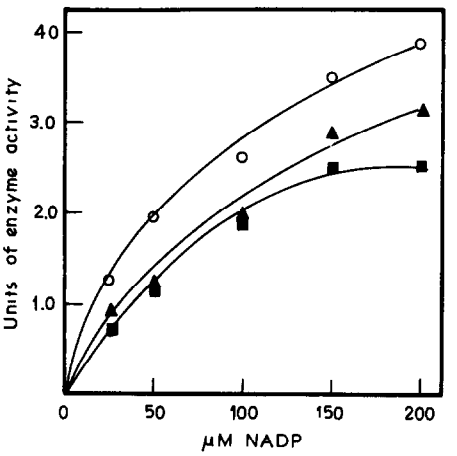

Fig. 8. Effect of NADPH on enzyme activity at varying concns of NADP and a fixed sub-saturating concn of malate. Details of the experiment as described in Fig. 3B except that in place of pyruvate, the following amounts of NADPH were included in the reaction mixture: ○, 0 (control); ▲, 0.05 mM; ■, 0.1 mM.

then be determined by the availability of NADP. It is thus conceivable that the observed response of the enzyme to the products and the substrates will afford an effective control mechanism for regulating utilization of the substrates, particularly when these are limiting, in accordance with cellular demands for products of the reaction. The observed effects of products on enzyme activity would still be physiologically relevant if, as in case of C_4 NADP-ME type plants, this enzyme also functions in the reproductive parts of C_3 plants to release CO_2 from malate for its subsequent refixation via the Calvin cycle.

EXPERIMENTAL

Materials. All biochemicals used in these investigations were from Sigma. 'Analar' grade chloride salts of various cations and sodium salt of anions, for examining the effect of metal ions on the enzyme activity, were purchased from B.D.H.

Enzyme extraction. Glumes (20 g) from developing wheat grains, were separated about 20 days after anthesis, from ears of field-grown wheat (*Triticum aestivum* L cv. WH-157) plants. These were homogenized in the presence of an acid washed sand in 100 ml of 0.05 M Tris–HCl (pH 7.3) containing 10 mM cysteine. The slurry was squeezed through four layers of muslin and the filtrate centrifuged at 10000 *g* for 20 min. The supernatant, referred to as crude extract, was used for further purification of the enzyme All the above operations were carried out at 4°.

Enzyme purification. NADP-malic enzyme in the crude extract was precipitated with solid $(NH_4)_2SO_4$ in the saturation range of 45–70%. After centrifugation at 10000 *g* for 20 min, the ppt was dissolved in 10 ml of the extraction buffer and dialysed against the same buffer for 10 hr with two changes of 1 l. each. The dialysed preparation was loaded onto a DEAE-cellulose column (2.5 × 30 cm) which had previously been equilibrated with the extraction buffer The sample was eluted by applying a batch-wise salt gradient. The elution buffer (250 ml) at each successive step contained 0.1, 0.2, 0 3 and 0.4 M NaCl. The bulk of the enzyme was eluted with buffer containing 0.3 M NaCl. Active fractions were pooled, concd to *ca* 4 ml by osmosis against solid polyethylene glycol and then loaded onto a Sephadex G-200 column (2.5 × 50 cm). The sample was eluted with the extraction buffer and fractions of 3 ml each were collected. The active fractions were then pooled and stored at 4°

Determination of M_r. M_r of the enzyme was determined by gel filtration through Sephadex G-200 (2.5 × 50 cm). Standard proteins used for calibrating the column were: catalase (232000), aldolase (160000), alcohol dehydrogenase (141000) and egg albumin (45000). M_r was also calculated from the relationship $M_r = 151\ (1.47 - V_e/V_0)^{1/3}$ [25] and agreed closely with that obtained from the standard curve prepared with the proteins of known M_r.

Enzyme assay. Activity of NADP-malic enzyme was determined spectrophotometrically at 30° by monitoring the rate of reduction of NADP at 340 nm [7]. Reaction mixture in a final vol. of 2 ml contained the following: Tris–HCl (pH 7.6), 150 μmol; malate, 10 μmol; Mn^{2+}, 2 μmol; enzyme preparation, 0.2 ml (10–15 μg protein) and NADP, 0.4 μmol. In preliminary experiments, it was established that the rate of NADP reduction was proportional to the time of reaction and amount of the enzyme. Any variation in the composition of reaction mixture has been described along with individual experiments. One unit of enzyme activity corresponds to one nmol of NADP reduced per min at 30°. Possible interference in activity by NADP-malate dehydrogenase present in the preparations, particularly during initial stages of purification was corrected from the reaction carried out in the absence of Mn^{2+}.

Protein estimation. Protein content of various fractions during column chromatography was monitored from their *A* at 280 nm. In all other samples, protein was estimated by Folin Phenol method [26].

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