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THE KINETIC MECHANISM OF PHOSPHOENOLPYRUVATE CARBOXYKINASE FROM *PANICUM MAXIMUM*

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Key Word Index—*Panicum maximum*; Poaceae; C4 metabolism; phosphoenolpyruvate carboxykinase; kinetic mechanism.

Abstract—The kinetic properties of phosphoenolpyruvate carboxykinase [ATP: oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.49] from leaves of the C4 grass *Panicum maximum* have been re-evaluated due to conflicting reports concerning pH optima, Mn^{2+} requirement of activity and K_m for different substrates among C4 and CAM plants. The native enzyme has a M_r of 360 000 and a strict requirement of Mn^{2+} for activity. The optimum pH for the carboxylation reaction is 6.8 and the K_m's for PEP, ADP and HCO_3^- are 1.46 mM, 60 μM and 3.7 mM, respectively; for the decarboxylation process the pH optimum is 8.4 and the respective K_m's for ATP and oxaloacetate are 21 and 61 μM. At pH 7.6 the maximum rate of the decarboxylation reaction is three-fold higher than that of the carboxylation process. Steady-state kinetic studies of the decarboxylation reaction under optimal conditions and the evaluation of product inhibition effects show that the kinetic mechanism of this enzyme is a sequential ordered bi-tri process, ATP being the first substrate entering the active site, followed by oxaloacetate, while CO_2 is the first substrate to emerge, followed by PEP and ADP.

INTRODUCTION

Phosphoenolpyruvate carboxykinase (PEPCK) is an essential enzyme in the decarboxylation step of the C4 metabolism of several groups of plants [1–3]; it also plays a central role in CAM metabolism [4]. The type of decarboxylating enzyme (PEPCK, NAD- or NADP-dependent malic enzyme) among C4 plants have been correlated with their water requirements [5]. The subcellular location of PEPCK been the subject of some controversy: while an initial report associated the enzyme to the chloroplast compartment [6], subsequent studies in several groups of plants, including *Panicum maximum*, have shown that the enzyme is cytosolic [7–9]. Concerning the kinetic properties of the enzyme, studies have been carried out in C4 plants such as *Panicum maximum* [10] and *Chloris gayana* [11] and also in a CAM plant, *Ananas comosus* [12]; however, contradictory results were obtained concerning the pH optima for activity and the K_m values for different substrates, making it difficult to evalaute their physiological significance. Finally, no study has been reported on the reaction mechanism of a plant PEPCK, or of any other ATP-dependent PEPCK. In this paper we report the results of a detailed kinetic study on the PEPCK of *Panicum maximum*, which has allowed us to determine the absolute kinetic constants of the enzyme and its reaction mechanism.

RESULTS

Enzyme purification and properties

PEPCK was purified 40-fold from young leaves of *Panicum maximum* essentially as described by Hatch and

Table 1. Purification of PEPCK from *Panicum maximum* leaves*

Fraction	Total protein (mg)	Total activity (μkat)	Specific activity (μkat/mg. protein)	Purification (fold)	Yield (%)
Homogenate	256	47.0	0.18	(1)	100
100 000 *g*: 1hr supernatant	135.00	61.0	0.45	2.45	129
40–50% $(NH_4)_2SO_4$	33.3	70.2	2.11	11.5	149
Sephacryl S-300	5.6	26.6	4.69	25.5	56.5
DEAE-cellulose	0.7	4.9	7.10	38.8	10.1

*The enzyme activity was measured in the carboxylation direction as described in the Experimental section.

Mau [11] for the *Cloris gayana* enzyme (Table 1). The purified enzyme is free from PEP-carboxylase but contains some activity of malic dehydrogenase. The apparent M_r of the enzyme, determined by gel filtration in Sephacryl S-300, is 360000. The enzyme has a strict requirement of Mn^{2+} for activity both in the carboxylation and the decarboxylation reactions, even in the presence of an excess of Mg^{2+}. The pH optimum for the carboxylation reaction is 6.8 and the activity drops to 50% of the maximum at pH 7.4; in the decarboxylation process the pH optimum is 8.4 and 50% of the maximum activity is observed at pH 7.8. This leads to the fact that at pH 7.6 the maximum velocity of the decarboxylation reaction is three-fold greater than that of the carboxylation reaction. Apparent K_m's for the substrates of the carboxylation reaction were obtained at optimal pH and in the presence of saturating concentrations of the cosubstrates from Hanes–Woolf plots (Fig. 1): the values for PEP, ADP and HCO_3^- are 1.46 mM, 60 μM and 3.7 mM, respectively. The maximum velocity under these conditions is 8 μkat/mg protein (obtained from the PEP plot). In the decarboxylation reaction (Fig. 2; pH 8.4) the apparent K_m's are 21 μM for ATP and 61 μM for oxaloacetic acid (OAA); the maximum velocity for the process is 24.4 μkat/mg protein (obtained from the OAA plot).

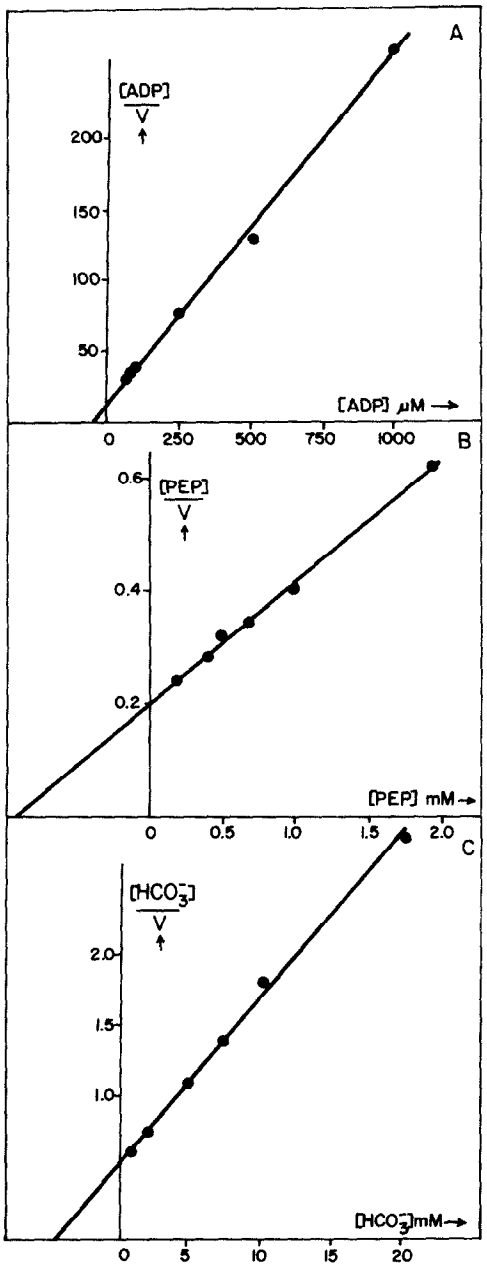

Fig. 1. Kinetics of *Panicum maximum* PEPCK in the carboxylation reaction. Hanes–Woolf plots of the activity of the enzyme as a function of ADP (A), PEP (B) and HCO_3^- (C) concentration. The concentration of the cosubstrates are: PEP 4 mM and HCO_3^- 20 mM for panel A, ADP 1 mM and HCO_3^- 20 mM for panel B and ADP 1 mM and PEP 4 mM for panel C. The carboxylation activity was measured as described in the Experimental section. Ordinate units are (mol/l)/(kat/mg protein) for panel A and (mol/l)/(mkat/mg protein) for panels B and C.

Reaction mechanism

We studied the reaction mechanism of *Panicum maximum* PEPCK in the decarboxylation direction using well established steady state kinetic methods [13–16]. Hanes–Woolf plots using ATP as variable substrate for different concentrations of OAA gave straight lines inter-

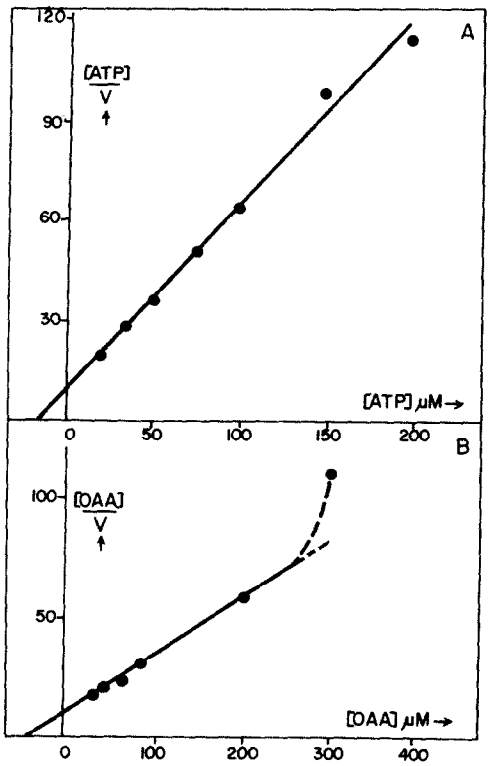

Fig. 2. Kinetics of *Panicum maximum* PEPCK in the decarboxylation reaction. Hanes–Woolf plots of the activity of the enzyme as a function of ATP (A) and OAA (B) concentration. The concentration of the cosubstrate is OAA 200 μM for panel A and ATP 400 μM for panel B. The decarboxylation activity was measured as described in the Experimental section. Ordenate units are (mol/l)/(kat/mg protein).

secting in the abscissa axis (Fig. 3A); the same situation was observed using OAA as variable substrate for different ATP concentrations (Fig. 3B). From the point of intersection of the lines in each plot the absolute Michaelis constant of the enzyme for the respective substrate (K_a or K_b in Fromm's notation, see ref. [15]) can be obtained; on the other hand, from the secondary plots of the inverse maximal velocities against the inverse of the substrate concentration (insets) the absolute maximum velocity of the process can be obtained. Also, the slopes of the secondary plots give an independent determination of the value of the absolute Michaelis constant of the co-substrate. The values obtained in this fashion showed internal consistency and are: K(ATP) = 21 μM, K(OAA) = 69 μM and V_m(abs.) = 24.6 μkat/mg prot. Product inhibition studies were used to further characterize the reaction mechanism: Fig. 4 shows the inhibition of the decarboxylation reaction produced by PEP (4A) and ADP (4B), using OAA as variable substrate and a fixed, saturating concentration of ATP [400 μM, 20 × K(ATP)]. It can be seen that PEP produces a non-competitive inhibition while ADP does not inhibit the reaction in the range of OAA concentrations where Michaelis–Menten (M-M) kinetics holds. At high OAA concentrations a clear deviation from M-M kinetics is observed, probably as a consequence of the formation of abortive complexes of OAA, enzyme and products. The inhibition of the decarboxylation reaction by PEP (5A) and ADP (5B), using ATP as variable substrate and a fixed, non-saturating concentration of OAA (150 μM) is shown in Fig. 5. It can be seen that PEP produces in this case a non-competitive inhibition, while the ADP inhibition is strictly competitive.

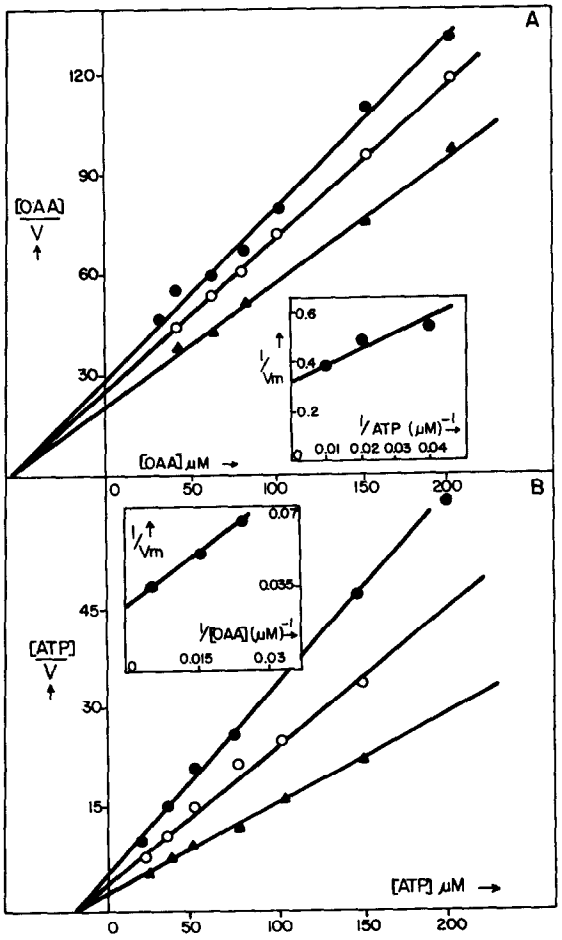

Fig. 3. Dependence of the kinetic parameters of *Panicum maximum* PEPCK in the decarboxylation reaction with the concentration of the co-substrate. Panel A: Hanes–Woolf plots of the enzyme activity as a function of OAA concentration with a fixed ATP concentration of (●) 25 μM, (○) 50 μM and (▲) 100 μM. Inset: Secondary plot of inverse maximal velocities, obtained from the slopes of the primary plots, as a function of the inverse of the ATP concentration. Panel B: Hanes–Woolf plots of the enzyme activity as a function of ATP concentration with a fixed OAA concentration of (●) 40 μM, (○) 60 μM and (▲) 200 μM. Inset: Secondary plot of inverse maximal velocities, obtained from the slopes of the primary plots, as a function of the inverse of the OAA concentration. Decarboxylation activity was measured as described in the Experimental section. Ordenate units are (mol/l)/(kat/mg protein) for the primary plots and (mg protein/μkat) for the secondary plots (insets).

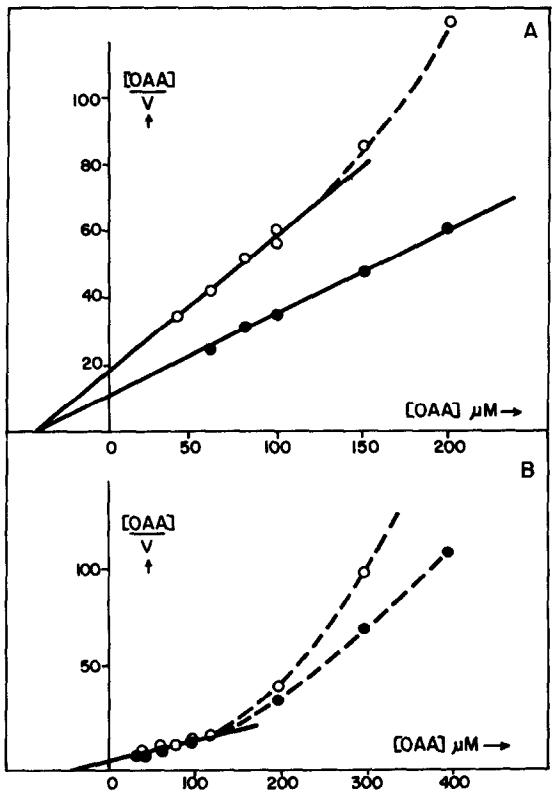

Fig. 4. Inhibition of the decarboxylation reaction catalysed by *Panicum maximum* PEPCK by PEP (panel A) and by ADP (panel B), for a fixed, saturating, ATP concentration (400 μM). Panel A: Hanes–Woolf plots of the enzyme activity as a function of OAA concentration in the absence (●) and presence (○) of PEP (4 mM). Panel B: Hanes–Woolf plot of the enzyme activity as a function of OAA concentration in the absence (●) and presence (○) of ADP (200 μM). Decarboxylation activity was measured as described in the Experimental section. Ordenate units are (mol/l)/ (kat/mg protein).

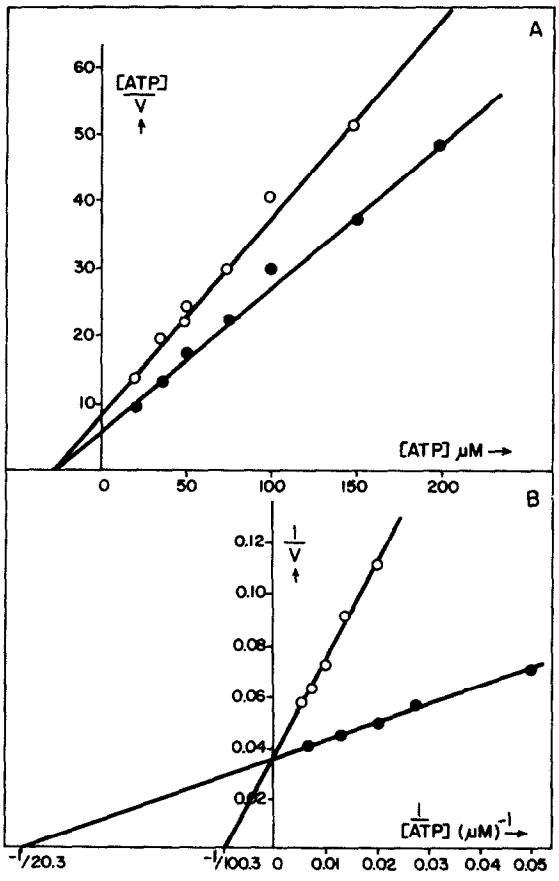

Fig. 5. Inhibition of the decarboxylation reaction catalysed by *Panicum maximum* PEPCK by PEP (panel A) and ADP (panel B), for a fixed, non-saturating, OAA concentration (150 μM). Panel A: Hanes–Woolf plots of the enzyme activity as a function of ATP concentration in the presence (○) and absence (●) of PEP (4 mM). Panel B: Lineweaver–Burk plot of the enzyme activity in the absence (●) and presence (○) of ADP (200 μM). Decarboxylation activity was measured as described in the Experimental section. Ordenate units are (mol/l)/(kat/mg protein) for panel A and (mg protein/μkat) for panel B.

DISCUSSION

The M_r of PEPCK from *Panicum maximum* confirms the existence of two divergent types of this enzyme in nature: the well known GTP-dependent enzymes present in vertebrates [17] and helminths [18] composed of a single 72000 polypeptide and the ATP dependent enzymes of bacteria [17], yeast [19], parasitic protozoa [20] and plants (this study), which have much higher M_r's and are probably composed of several subunits. The strict requirement of Mn^{2+} for activity found in the present study contradicts a previous report on the enzyme of the same species by Ray and Black [10]. One possible explanation for this discrepancy is the fact that all the assay media in the present study contained 0.1 mM EDTA to eliminate contaminating heavy metal ions which reduce the activity of the enzyme; in the assays of Ray and Black the chelant was not present and contaminating Mn^{2+} ions, which activate the enzyme by binding to a high affinity site in the PEPCK from different sources [20–22], could account for the residual activity. A strict requirement of Mn^{2+} for activity, in the presence of excess Mg^{2+}, has also been reported for the enzyme of another C4 plant, *Chloris gayana* [11] and in the helminth *Ascaris suum* [18]; however in vertebrates it is clear that Mn^{2+} can be replaced, albeit with less efficiency, by other divalent ions such as Mg^{2+}, Zn^{2+}, Co^{2+} and Cd^{2+} [17]. The alkaline pH optimum obtained in this study for the decarboxylation process catalysed by *Panicum maximum* PEPCK again does not agree with that reported in a previous study of the enzyme from the same source [10] and from the CAM plant *Ananas comosus* [12]; however alkaline pH optima for the decarboxylation reaction have been reported for the enzyme of *Chloris gayana* [11], *Trypanosoma cruzi* [20] and vertebrate sources [17]. The difference could be accounted for by the buffer system used: we have found that HEPES [4-2-(hydroxyethyl)-1-piperazinethanesulphonic acid] inhibits the decarboxylation reaction when compared with Tris in a pH-dependent fashion. The K_m values in the decarboxylation reaction found in this study agree with those reported in other plants, under different conditions; however, in the carboxylation reaction (all the studies have been carried at neutral or acid pH's) the K_m for HCO_3^- found in this study is three times smaller than that found by Ray and Black [10], although it is indistinguishable from the value found for the *Ananas comosus* [12], *Trypanosoma cruzi* [20] and *Ascaris suum* [18] enzymes. This value is, in turn, six to seven times smaller that that found for the vertebrate enzymes [17, 23]. In any case the different pH optima, maximal velocities and K_m values of the substrates for the carboxylation and decarboxylation reactions found in the present study provides further support to the notion that the net chemical flux that this enzyme catalyses *in vivo* in C4 plants is the decarboxylation of OAA [1, 2, 10, 11].

The lines intersecting in the abscissa in the Hanes plots for both ATP and OAA clearly indicate a sequential mechanism with $K_a = K_{ia}$, which means that product formation only occurs after the binding of both substrates to the active site and that the affinity of the enzyme for one substrate is independent of the concentration of the co-substrate [13, 15]. The product inhibition pattern exhibited by the PEPCK of *Panicum maximum* is only compatible with an ordered sequential process with ATP as the first substrate to enter the active site, followed by OAA; this would lead to CO_2 as the first substrate to leave the active site, followed by PEP and ADP (Fig. 6, see ref. [14]. The results of other studies on the kinetic mechanism of this enzyme are controversial: although a

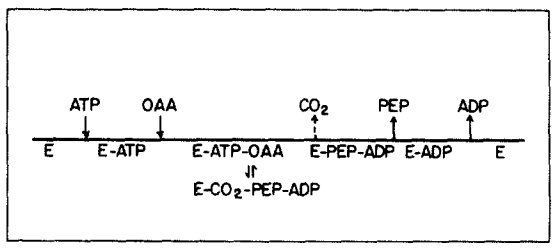

Fig. 6. Reaction mechanism proposed for *Panicum maximum* PEPCK.

study on the enzyme from chicken liver [24] indicated an ordered sequential process, with the same order of substrate entry and product release proposed by us for the *Panicum maximum* enzyme, Miller and Lane [25] concluded for the pig liver enzyme that the mechanism is a pseudo-random sequential process with PEP being the first substrate to enter the active site in the carboxylation reaction followed, in any order, by CO_2 and ITP and finally Jomain-Baum and Schramm [26] proposed a random sequential mechanism for the rat liver enzyme. In our case, the lack of inhibition by ADP when using OAA as substrate clearly rules out a random sequential process, while the proposed order of entry of the substrates (ATP first) is consistent with the specificity of this enzyme for adenine nucleotides [10, 11].

EXPERIMENTAL

Enzyme assays. PEPCK activity was determined in the carboxylation direction by coupling the formation of oxaloacetate to NADH oxidation by malate dehydrogenase at 25° [27]. The assay medium contained PIPES (1,4-piperazinediethanesulphonic acid) 50 mM, KCl 90 mM, 2-mercaptoethanol 5 mM, EDTA 0.1 mM, $MgCl_2$ 3 mM, $MnCl_2$ 3 mM, ADP 1 mM, PEP 2 mM, $KHCO_3$ 20 mM, 5 units of malic dehydrogenase, NADH 0.2 mM and the enzyme; the pH was adjusted to 6.8 with KOH. In the decarboxylation direction the activity was determined at the same temp. by following the disappearance of OAA at 280 nm according to ref. [28]; the reaction medium contained HEPES 50 mM, KCl 90 mM. 2-mercaptoethanol 5 mM, EDTA 0.1 mM, $MgCl_2$ 3 mM, $MnCl_2$ 3 mM, ATP 0.5 mM, OAA 0.4 mM and pyruvate kinase 2 units/ml, pH adjusted to pH 8.4. Under these conditions and in the OAA concn range used (0–400 μM) the molar extinction coefficient of this substrate was constant. For routine decarboxylation assays HEPES buffer was used instead of Tris, which gave a slighty higher activity (see above), to avoid the formation of insoluble Mn^{2+} complexes in this last medium.

pH-Dependence of activity. The pH-dependence of the carboxylation reaction was determined by measuring the enzyme activity in PIPES buffer as described above, varying the pH between 6.3 and 7.6. For the decarboxylation reaction Tris buffer was used and the pH range was from 7.4 to 8.6.

Determination of M_r. The M_r of the native enzyme was determined by gel filtration on a Sephacryl S-300 column (1.6 × 83 cm) equilibrated with HEPES 10 mM, KCl 50 mM, $MgCl_2$ 3 mM, $MnCl_2$ 3 mM, EDTA 0.1 mM, 2-mercaptoethanol 5 mM, pH 7.4 adjusted with KOH; the flow rate was 6.3 ml/hr and 4 ml fractions were collected. The column was calibrated with apoferritin (400 000), gamma-globulin (160 000) bovine albumin (67 000), whale sperm albumin (17 800) and cytochrome *c* (12 400).

Protein was determined by a modification [29] of the Folin phenol method of ref. [30], using bovine serum albumin as standard.

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