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Metabolism of *trans*-Aconitic Acid in Maize[1]

II. REGULATORY PROPERTIES OF TWO COMPARTMENTED FORMS OF CITRATE DEHYDRASE

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ABSTRACT

Kinetics of two molecular forms of K-dependent citrate dehydrase in maize (*Zea mays* L.) are reported. The isozymes, designated CD I and CD II, were found to be compartmented in mitochondria and cytosol, respectively.

CD I exhibited hyperbolic kinetics with respect to both citrate and potassium with K_m 2.3 and 12 millimolar, respectively. Maximum velocity was 0.38 micromole of *trans*-aconitic acid per minute per milligram protein. The pH optimum was 7.2. *trans*-aconitic synthesis by CD I is regulated by both citrate concentration and pH.

CD II exhibited hyperbolic kinetics with respect to citrate (K_m 0.6 millimolar) but sigmoidal kinetics with respect to potassium. *trans*-aconitic acid synthesis by CD II is regulated by potassium. This may account for the positive correlation between leaf potassium and *trans*-aconitic acid in certain grasses (Clark 1968 Crop Sci 8: 165).

trans-Ac[3] is the predominant organic acid in grasses (15). It accumulates in several species of lower plants and in bacteria (10). In *Pseudomonas* spp. *trans*-Ac is produced from *cis*-Ac via aconitate isomerase (10), whereas in maize (*Zea mays* L.) it is synthesized via K-dependent citrate dehydrase (1). Two molecular forms of this enzyme, designated CD I and CD II, were purified from coleoptile homogenates (1). The object of this study was to determine whether the kinetics of these isozymes (1) might account for the positive association between leaf K and *trans*-Ac accumulation in maize (2).

With CD II, potassium activation involves reduction of the K_m for substrate, whereas with CD I, potassium increases V_{max}. Kinetics of CD II at high K-low citrate concentrations appears to account for the positive correlation between leaf K and *trans*-Ac accumulation (2).

MATERIALS AND METHODS

Differential Centrifugation. Forty g of leaf tissue from 30-day-old corn (*Zea mays* L.) seedlings were ground in a mortar with an extracting buffer which was 0.25 M sucrose, 50 mM Tris HCl (pH 7.2) and 3 mM EDTA. The brei was filtered through cheese cloth, centrifuged at 1,000*g* for 5 min, 10,000*g* for 15 min and finally at 100,000*g* for 1 h. The activity of each isozyme was determined in 2 ml of the 100,000*g* supernatant after separation by gel filtration on Bio Gel A 5m column (1).

Purification of Mitochondrial CD I. Mitochondria in the 10,000*g* pellet were partitioned according to Douce *et al* (5). The pellet was suspended and washed twice by repeating the 1,000 and 10,000*g* steps. The final pellet was suspended in 2 ml of extracting buffer, layered over discontinuous sucrose gradient (4 ml each of 30 and 60% sucrose) and centrifuged 1 h at 33,000*g*. Mitochondria were collected at the sucrose interface, diluted with water to 8% sucrose and centrifuged at 10,000*g* for 15 min. After lysing in KCl buffer (50 mM Tris-HCl, [pH 7.2] 50 mM KCl) the lysate was centrifuged for 30 min at 33,000*g*. Activities of the isozymes in 2 ml of the above supernatant were determined after separation by gel filtration (1).

Kinetic Experiments. The influence of pH, temperature, K concentration, potential activators and inhibitors, and citrate concentration on *trans*-Ac production in 6-d-old corn seedlings was studied using isozymes purified as previously described (1).

Enzymic Assays. Citrate dehydrase activity was determined spectrophotometrically (2,13) at pH 7.2 unless otherwise noted. *trans*-Ac production was linear with enzyme concentration within the range of 5 to 30 μg/ml (data not shown). IDH (EC 1.1.1.42) was assayed according to Cox (4).

RESULTS AND DISCUSSION

Intracellular Compartmentation of Isozymes. Sixty-two % of the citrate dehydrase activity was found in the 100,000*g* supernatant (Table I). Gel filtration revealed that this activity was associated with CD II, indicating that this isozyme is a cytosolic protein. Absence of CD I activity in this fraction was taken as evidence for localization of this isozyme in another cellular compartment.

Thirty-one % of the citrate dehydrase activity was found in the 10,000*g* crude mitochondrial pellet (Table I). This fraction had the highest specific activity for both citrate dehydrase and IDH. The mitochondrial fraction was further purified by differential and density centrifugation, resulting in a 9-fold increase in specific activity for both IDH and citrate dehydrase (Table II). Mitochondrial lysate fractionated by gel filtration contained only CD I activity, indicating that this isozyme is preferentially localized in the mitochondria.

Time Course of *trans*-Ac Production. With CD I, there was a lag in *trans*-Ac production from citrate, manifesting a hysteretic response (8), which varied inversely with the amount of KCl present during assay (Fig. 1A). Accordingly, preincubation of the enzyme with KCl was a standard practice in all assays. In contrast with CD I, there was no lag in *trans*-Ac production from citrate with CD II (Fig. 1B).

Potential Activators and Inhibitors. Neither isozyme was activated by Tris or NH_4^+ (Table III). Alkali metals stimulated in the order $K^+ > Rb^+ > Na^+ > Cs^+ > Li^+$. This order is one of 11

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[3] Abbreviations: *trans*-Ac, *trans*-aconitic acid; *cis*-Ac, *cis*-aconitic acid; IDH, NAD^+-specific isocitrate dehydrogenase; CD I, mitochondrial form of citrate dehydrase; CD II, cytosolic form of citrate dehydrase.

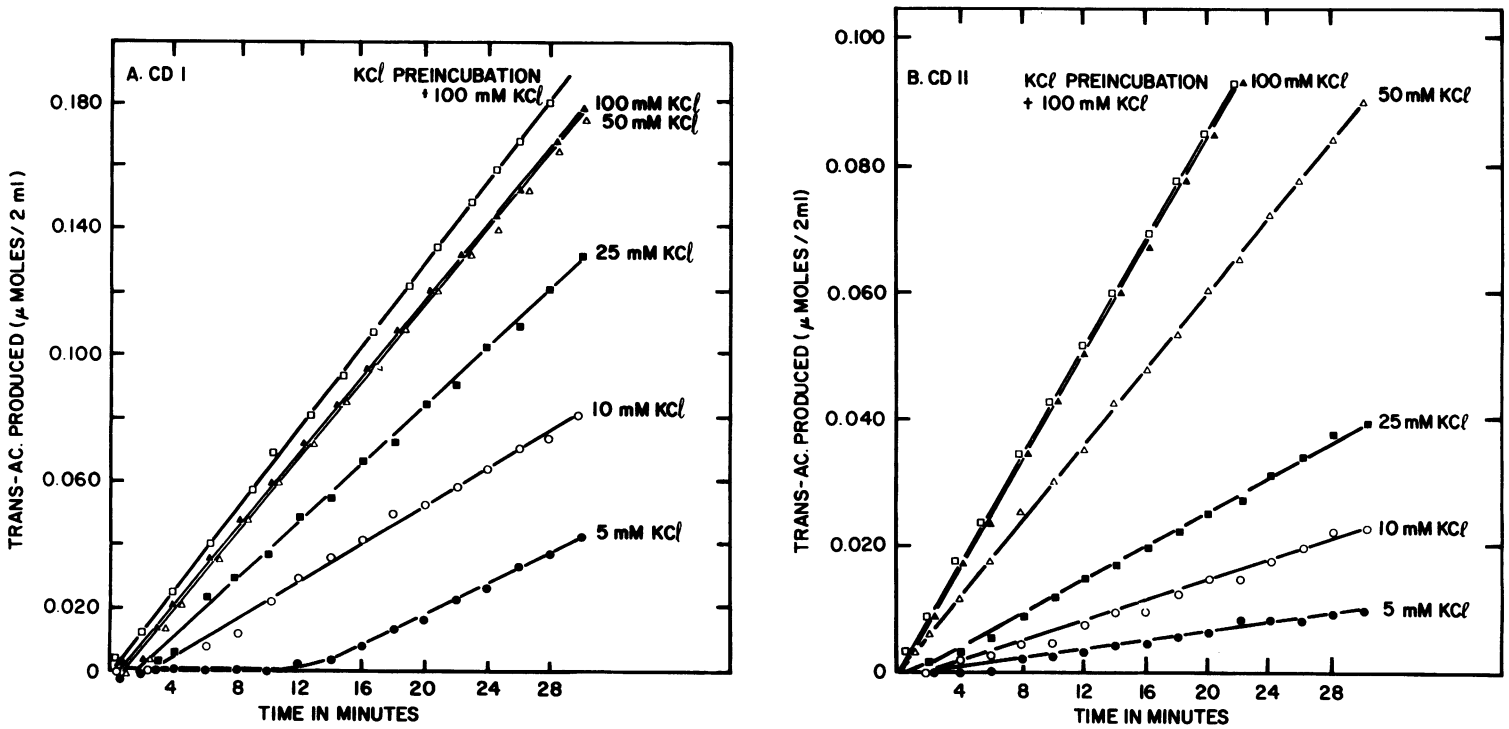

FIG. 1. *trans*-Ac production by CD I and CD II with time. Fifteen μg of CD I (A) or 25 μg of CD II (B) in 50 μl of 50 mM Tris-HCl (pH 7.2) were either added directly to 50 mM Tris-HCl, 10 mM citrate and 5, 10, and 100 mM KCl, or were incubated 30 min with 0.95 ml 50 mM Tris-HCl (pH 7.2) and 100 mM KCl prior to the addition of 1.0 ml 50 mM Tris-HCl, 100 mM KCl, and 10 mM citrate.

Table I. *Evidence of Compartmentation of Isozymes in 30-Day-Old Corn Leaf Tissue*

Fraction	IDH	Citrate Dehydrase	
	Specific activity	Total activity	Specific activity
	μmol/min·mg protein	*μmol/min*	*μmol/min·mg protein*
1,000*g* pellet	4.3	0.0066 (5)[a]	0.0004
10,000*g* pellet	42.8	0.0435 (31)	0.0052
100,000*g* pellet	8.9	0.0026 (2)	0.0011
100,000*g* supernatant	0.1	0.0869 (62)	0.0011

[a] Percentage of total activity.

Table II. *Copurification of Mitochondria and Mitochondrial Citrate Dehydrase*

Fraction	IDH Specific activity	Citrate Dehydrase	
		Total activity	Specific activity
	μmol/min·mg protein	*μmol/min*	*μmol/min·mg protein*
10,000*g* pellet	43	0.0435 (100)[a]	0.0052
Washed pellet	155	0.0405 (93)	0.0175
Gradient pellet	390	0.0352 (84)	0.0472
Lysate	ND[b]	0.0318 (73)	ND

[a] Percentage of the activity in the 10,000*g* pellet.
[b] Not determined.

theoretical rankings for binding of alkali metals to form ligands (6).

Klinman and Rose (10) found that both Fe^{2+} and glycerol increased the activity of aconitate isomerase. However, neither influenced the activity of citrate dehydrase, nor did BSA (2% w/v), 5 mM $MgCl_2$, or 5 mM EDTA affect activity. Maintenance of

Table III. *Influence of Monovalent Cations on Isozyme Activity with 10 mM Citrate as Substrate*

Cation	CD I	CD II
	μmol/min·mg protein	
50 mM Tris-HCl buffer	0	0
100 mM NH_4 Cl	0	0
100 mM KCl	0.340	0.138
100 mM RbCl	0.156	0.079
100 mM NaCl	0.031	0.021
100 mM $CsNO_3$	0.002	0.003
100 mM LiCl	0.002	0.001

Table IV. *Influence of Potassium on K_m of CD I for Citrate*

KCl	K_m	V_{max}
mM		*μmol/min·mg protein*
5	2.6	0.065
10	2.2	0.152
20	2.4	0.238
50	2.3	0.368

activity in the presence of EDTA, and the absence of stimulation by $MgCl_2$ indicates that citrate dehydrase has no divalent cation requirement. Activity of CD I was reduced from 0.350 to 0.250 μmol/min·mg protein in 1% 2-mercaptoethanol. Both enzymes were completely inhibited by 2% SDS (w/v) and 8 M urea.

K_m for K^+ and Citrate. CD I exhibited hyperbolic kinetics with respect to both K^+ and citrate, with K_m values 12 and 2.3 mM, respectively (Figs. 2A and 2B). The K_m for citrate was relatively constant with increasing KCl concentrations, whereas V_{max} increased from 0.065 to 0.368 μmol/min·mg protein (Table IV). The K_m of CD I for citrate (Fig. 2B) indicates that *trans*-Ac synthesis is regulated by citrate rather than by K^+. Inasmuch as this K_m falls within the range of citrate concentrations found in mitochondria (12,14) CD I would not be citrate saturated *in vivo*.

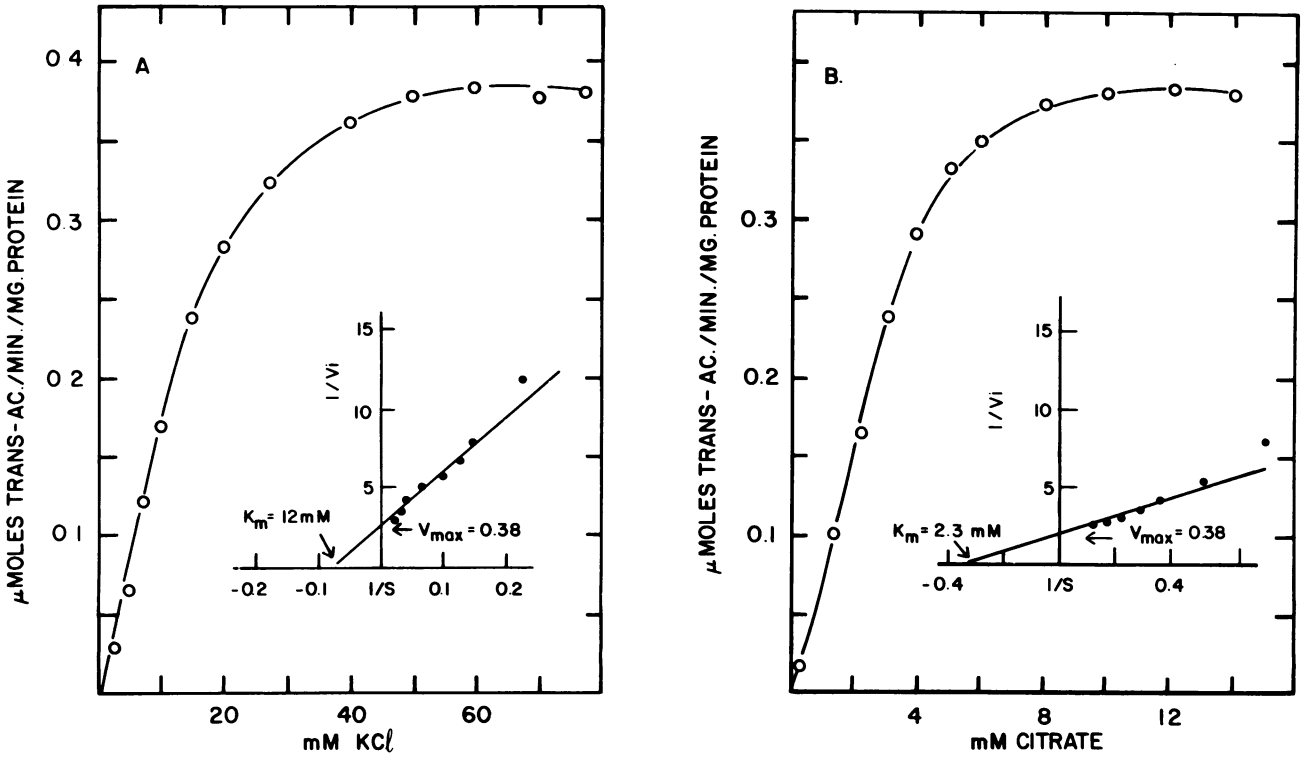

FIG. 2. K_m of CD I for K and citrate. A, Influence of K concentration at 10 mM citrate. B, Influence of citrate concentration at 100 mM KCl on *trans*-Ac production (pH 7.2) Lineweaver-Burk plots inserted.

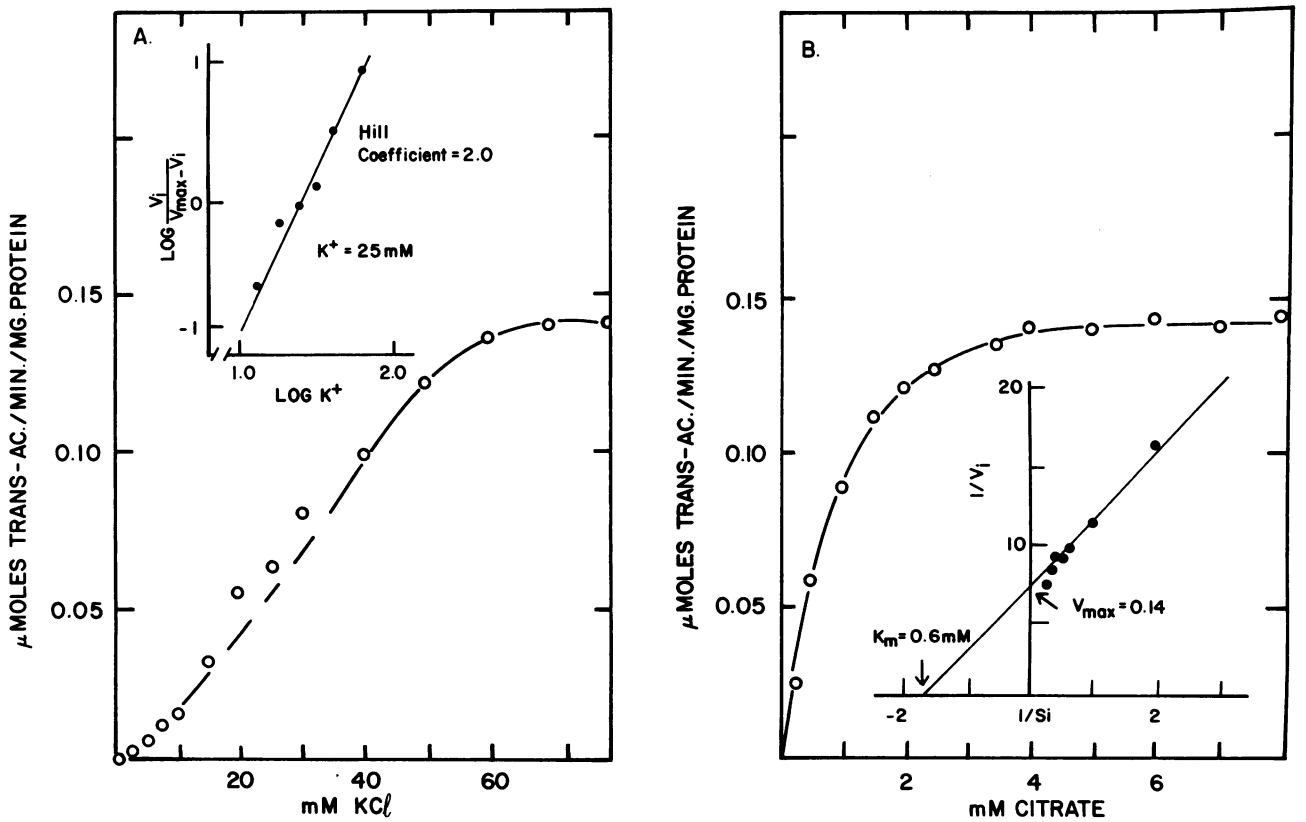

FIG. 3. K_m of CD II for K and citrate. A, Influence of K concentration at 10 mM citrate. B, Influence of citrate concentration at 100 mM KCl on *trans*-Ac production (pH 7.2). Hill and Lineweaver-Burk plots inserted in A and B, respectively.

Table V. *Influence of Potassium on Hill Coefficient and K_m of CD II for Citrate*

KCl Concentration	Hill Coefficient	K_m
mM		mM
10	8.1	2.6
25	3.0	1.5
40	1.8	1.2
100	1.0	0.6

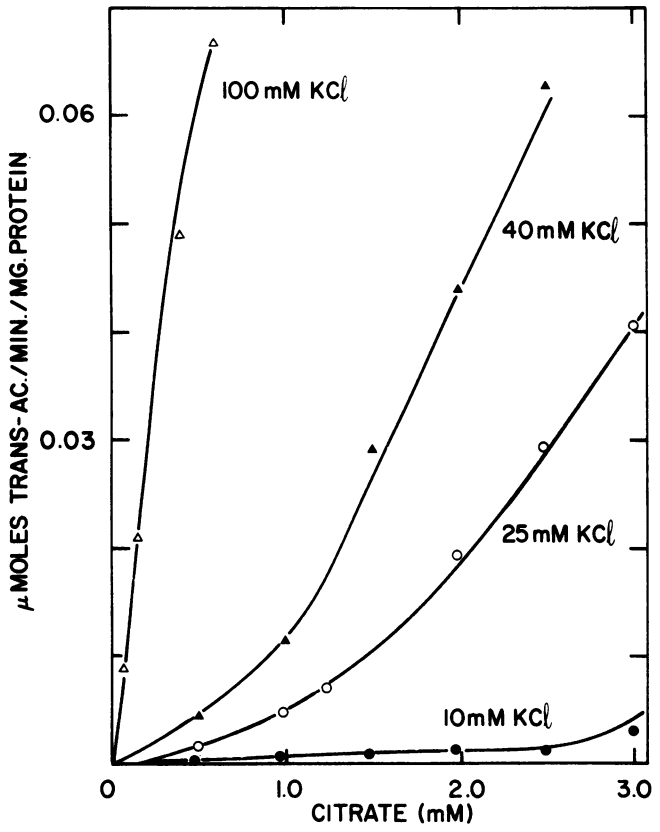

FIG. 4. Reaction rate of CD II as influenced by citrate concentration at 4 KCl concentrations.

It would, however, be K^+ saturated inasmuch as K^+ concentrations in plant tissue fall within the range of 25 to 50 mM (7,16).

In contrast with CD I, CD II exhibited sigmoidal kinetics with respect to K^+ at citrate saturation, but hyperbolic kinetics with respect to citrate at K^+ saturation (Fig. 3,A and B). The K_m and Hill coefficient for citrate decreased 4- and 8-fold, respectively, as KCl concentration was increased from 10 to 100 mM (Table V). Decreases in the Hill coefficient for citrate with increasing K^+ does not imply that the number of binding sites for citrate was decreased. Rather it suggests that the cooperativity between binding sites was altered. This suggests that *trans*-Ac synthesis by CD II is regulated by K^+. Increasing K^+ concentration decreased the K_m for citrate to a physiologic level; approximately 0.5 mM (14).

With 10 mM KCl, the activity of CD II at 0.5 mM citrate was very low (Fig. 4), however, with 100 mM KCl, activity approached 0.067 μmol/min·mg protein. The marked effect of K^+ at physiologic levels of citrate (0.5 mM) is clearly due to a reduction in the K_m for citrate (Table V). This relationship may account, in part, for the positive correlation between leaf K^+ and *trans*-Ac (2), and for the marked accumulation of this acid early in the ontogeny of maize when K^+ concentration is maximal (3).

Influence of Temperature and pH. The temperature optimum for each isozyme was 55°C. The pH optimum for CD I was narrowly defined at 7.2, whereas the optimum for CD II ranged between 7.0 and 8.0 (Fig. 5). The narrow optimum pH range for CD I suggests that mitochondrial pH governs CD I activity. Thus, when respiration rate is high, *trans*-Ac synthesis via CD I would be low inasmuch as respiration favors H^+ expulsion and cation influx (9). According to the Mitchell hypothesis (11), OH^- accumulates within the mitochondria during H^+ expulsion. From Figure 5, it is seen that if mitochondrial pH changes ±1 unit from the optimum (pH 7.2), CD I activity would be expected to decrease 50%.

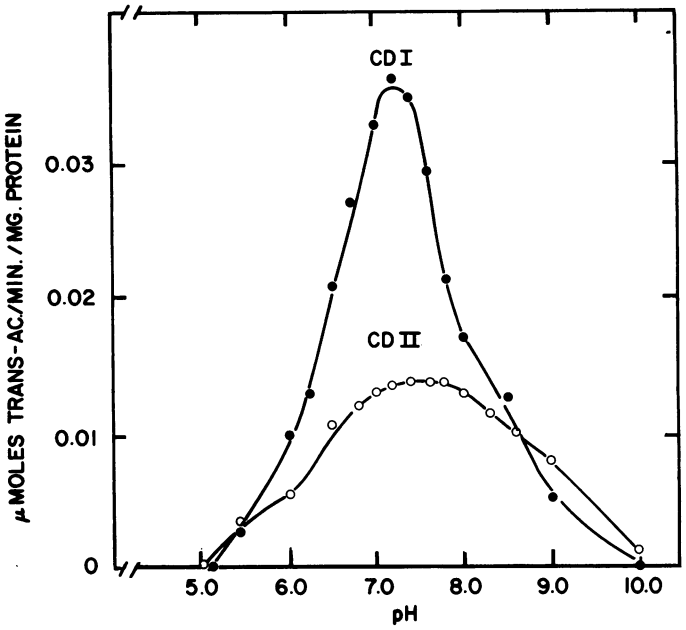

FIG. 5. pH profiles of CD I and CD II. *trans*-Ac production by CD I (□) and CD II (○) as influenced by pH using 10 mM citrate as substrate at

CONCLUSIONS

Kinetics of CD I and CD II were examined to determine whether either of the isozymes might be regulated by K^+ *in vivo* and thereby account for the positive association between leaf K^+ and *trans*-Ac accumulation (2). It was necessary to establish intracellular compartmentation of the respective isozymes in order to interpret the kinetics. We found evidence that CD I and CD II were respectively mitochondrial and cytosolic proteins (Tables I and II). Kinetics suggest that CD I is not regulated by K^+ *in vivo* since K_m for K^+ was low (Fig. 2). In contrast, increasing K^+ decreased both the K_m and Hill Coefficient of CD II for citrate, resulting in a marked increase in enzyme activity. CD II may therefore account for the major portion of *trans*-Ac accumulation under high K fertility (2).

Due to the apparent association between *trans*-Ac and grass tetany (15), it should be established whether or not it is possible to reduce *trans*-Ac levels in grasses by reducing the rate of K fertilization. In general, K deficiency occurs when tissue K^+ is less than 25 mM (7). Nevertheless, at this concentration, CD I activity is relatively high (Fig. 2A). In contrast, CD II activity at physiologic levels of citrate (0.5 mM) is almost nil (Figs. 3B and 4). Thus, even though CD II accounts for approximately 60% of the citrate dehydrase activity (Table I), *trans*-Ac synthesis is not completely disrupted at low K^+ levels. With severe K^+ stress in maize (Schultz and Teel, unpublished) *trans*-Ac accumulation was reduced only 40%, therefore, reducing K^+ fertility may not be a plausible solution.

Differences in citrate dehydrase activity among maize cultivars

(Brauer, unpublished) has been observed. Consequently, there may be opportunities through breeding and selection to reduce *trans*-Ac levels in grasses.

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