Role of Aconitate Isomerase in *trans*-Aconitate Accumulation in Plants

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Wheat seedlings (*Triticum aestivum*) grown on single salt solutions were analyzed for *trans*-aconitate and aconitate isomerase to determine the possible relationship between them and to determine if they might be related to the incidence of grass tetany. The *trans*-aconitate activity at the end of a two-day growth period and changes in *trans*-aconitate activity during this period varied widely and were related to the cation but not the anion of the single salt solution. Aconitate isomerase activity did not vary as much as *trans*-aconitate or change in *trans*-aconitate content, and was not closely correlated with the *trans*-aconitate content. Analysis of diverse species and tissues for aconitate isomerase and *trans*-aconitate showed that the presence of aconitate isomerase was necessary for appreciable *trans*-aconitate accumulation. We conclude that the activity of aconitate isomerase does not control *trans*-aconitate content but that its presence is necessary for any significant accumulation of *trans*-aconitate.

Keywords: *Wheat; grass tetany; aconitate; aconitate isomerase; organic acids*

INTRODUCTION

We have been interested in the accumulation of *trans*-aconitate (TA) in plants because of its possible role in the etiology of grass tetany syndrome (Orioli and Thompson, 1990). Grass tetany syndrome (Littledike et al., 1983) is a general term for several nutritional diseases (e.g., grass tetany, wheat pasture poisoning, milk fever) of ruminants that are characterized by a deficiency of Mg and/or Ca in the blood and urine of affected animals (Rendig and Grunes, 1979; Littledike et al., 1983). Several factors are responsible for the Mg and/or Ca deficiency including marginal levels of Mg and Ca in the feed, high levels of K and N, long-chain fatty acids (in lipids), and/or TA in the forage (Rendig and Grunes, 1979; Littledike et al., 1983). The problem is particularly prevalent during pregnancy and lactation when the mother supplies Mg and Ca to the developing fetus and milk. In their efforts to determine the cause of a particularly severe grass tetany problem, Stout and co-workers (Burau and Stout, 1965; Stout et al., 1967) analyzed a number of range forages and found that many contained high contents of TA. In a wide variety of range plants, they found many plants contained 1% TA on a dry weight basis and some species contained considerably more. Because of the ability of TA to chelate divalent cations, they concluded that one of the causes of grass tetany is the accumulation of TA in forages.

With the goal of reducing the grass tetany problem, we have investigated factors affecting the accumulation of TA in plants, mainly wheat seedlings (Orioli and Thompson, 1990). Utilizing wheat seedlings, we showed that TA accumulation to 1% dry weight can be induced by growth for 5 days on a K_2SO_4 solution but not in seedlings grown on $CaSO_4$ (Orioli and Thompson, 1990). Furthermore, it was shown that TA did not accumulate in the roots and that TA was not formed in the roots and translocated to the leaves (Orioli and Thompson, 1990).

The in vivo pathway for TA formation in wheat is uncertain. Brauer and Teel (1981a,b) found an enzyme, citrate dehydrase, in maize (*Zea mays* L.) that converts citrate to TA, but we have been unable to demonstrate this enzyme in wheat. We have observed an active aconitate isomerase (AI) (Thompson et al., 1990) that could facilitate the reversible isomerization of *cis*- and *trans*-aconitate. Consequently, we believe that, in wheat, TA is formed from *cis*-aconitate (CA) by the action of AI. CA is well known as an intermediate between citrate and isocitrate in the Krebs citric acid cycle (Glusker, 1971).

The fact that TA and malate accumulated to a much higher level in leaves of wheat seedlings grown on K_2SO_4 than in seedlings grown on $CaSO_4$ (Orioli and Thompson, 1990) indicated that the accumulation of TA might be a response to the rapid cation (K^+) and slow anion (SO_4^{2-}) uptake into plants. It has been known for many years that plants exposed to salts in which the cation is absorbed more rapidly than the anion will accumulate organic acids (Ulrich, 1941; Hiatt, 1967).

These facts led us to ask two questions. First, is the accumulation of the TA the result of rapid cation uptake coupled with slow anion uptake? Second, what is the relationship between TA and AI content?

In the experiments reported here, we grew wheat seedlings in a number of buffered single salt solutions and measured TA and AI. The single salts were chosen on the basis of published evidence for different rates of cation and anion uptake. AI was determined because we believed that it was the enzyme responsible for TA accumulation since we have been unable to find evidence for citrate dehydrase in wheat. We also measured TA and AI in species other than wheat to determine whether there was a correlation between the two parameters. Our results indicate that the presence of appreciable AI is essential for TA accumulation but that

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the amount of AI present does not control the extent of TA accumulation.

MATERIALS AND METHODS

Chemicals. Organic acids were purchased from Sigma Chemical Co., St. Louis, MO. Dowex-1, 200−400 mesh, was bought from Aldrich Chemical Co., Milwaukee, WI. All salts and solvents used were analytical grade.

Source of Plant Material. Wheat seed (*Triticum aestivum* L. cv. Tascosa) was obtained from Richardson Seed Co. Vega, TX.

A number of species (other than wheat) and tissues were analyzed for TA and AI. Storage tissues (e.g., root, bulb, tuber) were obtained from a local market. The vegetative tissues were obtained from plants grown outdoors or in a greenhouse with normal fertilization. The non-wheat species were chosen to represent most of the major higher plant families and one lower species, *Equisetum arvense* L.

Equipment. The HPLC column used for the analysis of wheat extracts was a C18 column (Beckman ODS-Ultrasphere) (25 × 0.46 cm). A guard column (4.5 × 0.46 cm) of the same packing material was employed. The analytical setup utilized Beckman (Altex-110B) pumps and controller, model 420 (San Ramon, CA); Hewlett-Packard multiwavelength detector-series 1050 (Palo Alto, CA) and a Spectra-Physics integrator-model SP4270 (Palo Alto, CA). For measurement of TA in the non-wheat species, two tandem ION-300 columns from Interaction Chromatography Co. (San Jose, CA) were utilized.

Growth and Handling of Wheat Seedlings. Wheat seeds (45 g) were submerged in aerated deionized water with several changes overnight at room temperature. Then the seeds were spread out on four layers of cheesecloth (13 × 24 cm) over an aerated solution (3 L) of 0.5 mM $CaSO_4$ and solution pH was buffered at 6.0 ± 0.1 with 5 mM tris-mes (Miyasaka et al., 1988). Seedlings were grown under continuous cool white fluorescent bulbs at a photon flux of 100 μmol m^{-2} s^{-1}. The cheesecloth dipped into the solution and acted as a wick to keep the seeds wet. After 5 days, the solution was replaced with an experimental solution (see below). Forty-eight hours later the seedlings were harvested. At harvest, the pH of the remaining solution was measured and in all cases was 6.0 ± 0.1. The shoots were excised, weighed, and cut into 1-cm pieces. A 6-gm portion was weighed and immediately frozen in liquid nitrogen for AI determination (see below). The remainder of the leaf segments were stored at −20 °C in 95% ethanol until they were ground and extracted (see below).

Experimental Treatment of Wheat Seedlings. As indicated above, 5-day old seedlings were placed in various well-aerated buffered single salt solutions for 2 days before harvest. In addition to the tris-mes buffer to minimize changes in pH, each experimental growth solution contained one salt. The following salts were included at 10 mM: KCl, K-mes, NaCl, Na-mes, and tris-Cl. Other salts; K_2SO_4, RbCl, Na_2SO_4, $CaCl_2$, $CaSO_4$, Ca-mes, or tris-sulfate, were included at 5 mM. Rb_2SO_4 solution was used at 2.5 mM. Note that all salt solutions were used at concentrations of 10 meq/L except for Rb salts in which the concentrations were 5 meq/L to avoid toxicity that is evident at 10 meq (L. V. Kochian, personal communication). In control treatments, seedlings were transferred to fresh buffered $CaSO_4$ solution. The full nutrient solution treatments were transferred to full nutrient solution (Madison et al., 1976) after 5 days in $CaSO_4$ solution. All treatments were run in duplicate.

Determination of TA. Wheat leaves were extracted with 50% ethanol at low temperatures as described (Orioli and Thompson, 1990). For most samples for which there was sufficient TA and the method for measuring TA was sensitive enough that TA could be measured directly on the alcoholic extract. For those samples for which the TA peak was small, the extract was dried in a desiccator at 0−4 °C, and the residue was dissolved in water and then filtered. TA was measured by reverse phase HPLC chromatography (Tusseau and Benoit, 1987) using absorbance at 210 nm to detect the TA. Quantitation was obtained by using an integrator to measure the area under the peaks by comparison with a standard curve prepared with pure TA. No attempt was made to measure other organic acids.

The TA in plant species other than wheat was measured by an ion exclusion method (Togami et al., 1990). Two columns (300 × 4.6 mm) of ION-300 were connected in tandem and maintained at 0 °C. Sulfuric acid (1.5 mM) was pumped through the column at 0.2 mL/min, TA was detected by absorbance at 210 nm and quantitated with an integrator.

No CA was detected when TA was chromatographed, which shows that there was no isomerization of aconitate under these conditions. Since TA was identified by its elution time, evidence that a given peak was due to TA was obtained by isolating anions and rechromatography. Anions were isolated by adsorption on an anion exhange resin (e.g., BioRad AG1-X8) and elution with 6 M HCl.

Measurement of AI. Frozen wheat leaves (6.0 ± 0.1 g) were ground and extracted with buffer (0.1 M tris-acetate, 1 mM mercaptobenzothiazole, 5 mM dithioerythritol, pH 7.5) and insoluble material was removed by centrifugation. The proteins were precipitated with 70% saturated ammonium sulfate, and the precipitate was isolated by centrifugation and dissolved in the same buffer. The resultant solution was desalted on a column of Sephadex G-25 and assayed for AI as described (Thompson et al., 1990). The ^{3}H-TA [0.25 μCi of 27.7 μCi/μM] which was used as substrate was prepared in this laboratory according to our published method (Thompson et al., 1990) and the released tritium was measured by liquid scintillation.

Since the growth treatments with single salts were conducted over a considerable period of time (one salt per week), a control incubation was included with the analysis of each batch of seedlings as follows. A large batch of wheat leaf extract was prepared, frozen with liquid nitrogen in 0.5-mL aliquots, and stored at −80 °C. With each sample, one tube was thawed and assayed for AI. This activity was used to correct for any possible week-to-week variations. There was no evidence that the frozen extract lost activity at −80 °C. In addition, an aliquot of the frozen wheat extract was added to an aliquot of the test extract from the non-wheat species (Table 4) to test for the presence of inhibitors or activators; in no case was there evidence of an inhibitor or activator.

RESULTS

Wheat seedlings grown on various single salt solutions were analyzed for TA and AI. The single salts were chosen to represent a range of cations and anions which vary with their rate of uptake by roots. For example, K and Rb were chosen as rapidly absorbed cations, whereas calcium and tris (Ferguson et al., 1980) were chosen as representatives of slowly absorbed cations. Several Na salts were included because Na often behaves differently from K and Rb at least with respect to uptake by roots at higher concentrations (Epstein et al., 1963). Sulfate (Hiatt, 1967) and mes (Ferguson et al., 1980) were used as examples of slowly-absorbed anions while chloride served as an example of a rapidly-absorbed anion (Epstein et al., 1963).

Table 1 presents the results of analyses of wheat seedling shoots for TA and AI grown on single salt solutions for 2 days. The results of root analyses are not presented because previous work showed that wheat roots have low contents of TA and AI (2% of AI and 0.08% of TA) compared to the shoots (Orioli and Thompson, 1990). These analyses show that the AI contents of treated seedlings, expressed on a fresh weight basis, did not vary as widely (max/min = 1.89) as the TA content (max/min = 47.1). Because there was considerable growth during the 2-day treatment, the changes in TA (CTA) and AI during treatment were calculated. The calculations of change in AI gave the same pattern as AI level and therefore are not included in Table 1.

Table 1. *trans*-Aconitate and Aconitate Isomerase Contents and Change in *trans*-Aconitate Content in Shoots of Wheat Seedlings Grown on Various Single-Salt Solutions for 2 Days[a]

treatment	TA (μmol/gfw)	aconitate isomerase (activity U[b]/gfw)	change in TA (μmol/ 6 g of sample)
control, 5 day[c]	2.24 ± 0.62	33.0 ± 7.1	
control, 7 day[d]	0.37 ± 0.20	14.3 ± 0.78	−20.6 ± 8.6
KCl	22.6 ± 5.8	26.8 ± 9.2	807 ± 126
K_2SO_4	21.8 ± 3.0	26.6 ± 0.14	719 ± 16
K-mes	22.4 ± 0.69	33.7 ± 10.8	847 ± 292
RbCl	10.7 ± 0.023	27.9 ± 2.6	375 ± 23
Rb_2SO_4	19.0 ± 4.9	28.9 ± 8.6	511 ± 215
NaCl	1.37 ± 1.7	32.9 ± 0.57	6.7 ± 54
Na_2SO_4	5.50 ± 3.8	22.2 ± 2.0	109 ± 72
Na-mes	5.06 ± 0.23	19.1 ± 2.0	111 ± 4.1
$CaCl_2$	0.48 ± 0.21	21.6 ± 0.42	−19 ± 7.5
$CaSO_4$	0.57 ± 0.34	24.2 ± 2.8	−16 ± 9.0
Ca-mes	0.93 ± 1.0	20.1 ± 2.2	−2.7 ± 35
tris-Cl	1.56 ± 0.26	17.8 ± 2.9	3.0 ± 13
tris-SO_4	1.64 ± 1.5	27.2 ± 16.7	14.4 ± 44
nutr soln[e]	8.07 ± 0.67	40.3 ± 0.71	249 ± 28

[a] Mean ± standard deviation. [b] U denotes units of AI activity which are nanomoles of TA isomerized to CA in 2 h under standard conditions (Thompson et al., 1990). [c] Control, 5 day, refers to seedlings sampled at the time other seedlings were transferred to single-salt solutions. [d] Control, 7 day, refers to seedlings transferred for 2 days to the same solution as used during the first 5 days. [e] Five-day-old seedlings were transferred to a complete nutrient solution (Madison et al., 1976) for 2 days.

Table 2. Analysis of Variance of Change in *trans*-Aconitate for All Wheat Seedling Treatments

source	DF	mean square	*F* value	probability
cation	4	329600	38.14	0.0001
anion	2	8320	0.96	0.41
catxan	6	9800	1.13	0.40
total AI	1	56100	6.49	0.0255
error	12	8640	–	–

In the subsequent text, only CTA (during the 2-day treatment) is discussed since the CTA gave essentially the same results as total TA content and because CTA better reflects the effect of the single salts during the 2-day treatment.

To learn more about the relationship between CTA and AI, these parameters were plotted for all the treatments (data not shown). Overall, there was a positive correlation between CTA and AI ($CTA = -241 + 0.62\ AI$; $p < 0.01$; $r^2 = 0.38$). Since Table 1 shows that wheat seedlings grown on Na, Ca, and tris salts have low CTA and a range of low AI contents, the analyses from these treatments indicated that there was little correlation between CTA and AI in these treatments ($CTA = 0.00 + 0.032\ AI$; $p = 0.09$; $r^2 = 0.18$).

Examination of the relationship between individual cations and AI also showed no significant correlation. When CTA and AI were subjected to ANOVA (SAS) with CTA as the dependent variable, AI as a covariate, and cation and anion as class variables, it was found that CTA was affected by cation but not by anion. In addition, there was no interaction between cation and anion, and a significant probability that CTA is related to AI (Table 2). Large *F* values for cations ($F = 38.14$, $p = 0.001$) and for the covariate, AI ($F = 6.49$, $p = 0.0255$), were obtained indicating real differences among cations and a significant relation with the covariate. To gain further insight into cation−CTA interaction, these parameters were plotted for each anion (plots not shown) and showed a similarity in response and hence little evidence of interaction.

Table 3. Change in the *trans*-Aconitate Content and Aconitate Isomerase Content of Wheat Seedling Shoots Grown on Single-Salt Solutions Collated with Respect to Individual Cations or Anions[a]

cation or anion	average change in TA (μmol/6 g of sample)	average aconitate isomerase activity (U/gfw)
potassium	791a	1111a
rubidium	443b	949a
sodium	76c	712a
calcium	−13c	683a
tris	9c	632a
chloride	242a	833a
sulfate	271a	791a
mes	283a	854a

[a] Averages with the same letter are not statistically different using Waller−Duncan *k*-ratio *t* test and Scheffe's test ($p < 0.05$) from other averages in the same group (i.e., anion or cation).

Table 3 confirms and extends the results shown in Table 2 demonstrating no specific anion effect on CTA and that AI content of wheat shoots was not affected by either cation or anion. Table 3 also shows that K salt-treated seedlings had a higher CTA than Rb salt-treated seedlings and that both had a higher CTA than Na, Ca, and tris salt-treated seedlings. It is noteworthy that the CTA in shoots grown on K salts was higher than that in Rb salt-treated shoots (Table 3) even though K and Rb are generally considered to have similar rates of uptake in plants (Lauchli and Epstein, 1970). Possibly, this difference was due to the lower Rb concentration which was employed to avoid toxicity. The fact that the CTA of Na salt-treated shoots was less than that for K salt- and Rb salt-treated shoots supports the generally accepted notion that in most plants Na uptake is different from that of K and Rb (Epstein et al., 1963). It is not so surprising that the CTA of Ca salt-treated shoots was markedly less than that of K salt-treated shoots. The calcium ion is not readily absorbed (Hiatt, 1967; Marschner, 1986) so that excess cation uptake and a transient cation excess (inducing TA accumulation) is unlikely to occur. These results in toto indicate that some property of cations other than rate of uptake may govern TA accumulation. In a separate experiment, we tested the possibility that K might activate AI and thus influence TA content, but found that K had no effect on AI activity (data not shown).

Since the experiments with wheat indicated that there was low correlation between TA or CTA and AI content when seedlings were grown on Na, Ca, or tris salts, we decided to examine a possible correlation by measuring TA and AI in diverse plant species and tissues. Stout and co-workers (Burau and Stout, 1965; Stout et al., 1967) analyzed the leaves of a number of range forage plants for TA content and found that there was a wide variation in the TA content. Since it seemed possible that this variation was due to AI content, we analyzed the TA and AI in a number of taxonomically diverse plant species (representing different families) and in various tissues. The analyses presented in Table 4 show that there was a marked difference in the TA and AI content among species and tissues. Statistical analysis showed that there was a positive correlation between TA and AI ($r^2 = 0.74$; $p = 0.001$). Among the higher plant species analyzed, a high AI activity (> 5.0 U/gram of fresh weight) was restricted to grasses. Clearly, high AI activity was correlated with a high TA content (see the values for *Triticum*, *Zea*, and *Agropyron* leaves). Shoots of wheat seedlings, grown on a complete

Table 4. *trans*-Aconitate and Aconitate Isomerase Activity of Several Species and Tissues[a]

species	tissue	TA content[b] (μmol/gfw)	aconitate isomerase activity[b] (U/gfw)
Triticum aestivum	shoot	8.1	40.3
	root	0.011	0.960
Zea mays	leaf	155.	58.8
	root	15.2	10.8
Agropyron cristatum	leaf	0.3	15.2
	root	trace	4.0
Glycine max	leaf	trace	2.1
Spinacia oleracea	leaf	trace	0.38
Raphanus sativus	root	trace	ND
Narcissus tazetta	leaf	ND	0.11
Lactuca sativa	leaf	ND	4.0
Solanum tuberosum	tuber	ND	0.18
Cucumis sativus	leaf	ND	0.15
Malus domestica	leaf	trace	ND
Equisetum arvense	shoot	1.0	0.16
Brassica oleracea			
Var. botrytis	leaf	ND	0.26
	immature inflorescence	trace	ND
Araucaria excelsa	leaf	trace	ND

[a] In addition to the species and tissues listed in the table, no detectable TA or AI was observed in the following: *Triticum aestivum* mature seed, *Narcissus tazetta* bulb, *Solanum tuberosum* tuber, *Cucumis sativus* fruit, *Malus domestica* fruit, *Daucus carota* leaf and root, *Elaeis oleifera* leaf blade, *Brassica oleracea* (var. botrytis) immature inflorescence, and *Apium graveolens* leaf and root. [b] ND = not detected TA < 10 nmol/gfw, AI < 100 μU/gfw. Trace means that there was some present but that it was not quantifiable.

nutrient solution, had a much higher TA and AI content than roots from the same plants (Table 4). With maize, the difference between shoot and root was in the same direction but was not as large. These results led us to discard our initial hypothesis that TA accumulation in wheat was just a result of rapid cation uptake coupled with slow anion uptake and to conclude that appreciable TA accumulation requires AI but that AI does *not* control TA level.

DISCUSSION

The seedling experiments indicate that CTA is related to more rapid cation than anion uptake but that other factors are also involved. This statement is illustrated by the results of CTA and TA content of seedlings grown on KCl and K_2SO_4. It is generally considered that the chloride ion is more rapidly absorbed than the sulfate ion (and this is true in barley [Hiatt, 1967]) and yet the CTA of KCl-grown seedlings is essentially the same as that of seedlings grown on K_2SO_4 (Table 1). Hence, these results indicate that TA content is not solely regulated by an excess of cation over anion uptake. On the other hand, the data do indicate that the nature of the cation is important and a possible explanation is presented below. The CTA values were cation specific (Table 3). The differences in CTA elicited by the monovalent cations (K, Rb, Na, and tris) indicate that rate of uptake is not the only criterion for TA accumulation. Because species and tissues with negligible AI do not accumulate TA (Table 4), it appears that TA content was dependent on AI activity.

We realize that a lack of an effect of AI content on TA content or CTA for some of the wheat treatments (seedlings grown on Na, Ca, and tris salts) does not prove that there is no effect of AI on TA content or CTA. For example, the TA content of various plant species (Table 4) and of forage plants (Burau and Stout, 1965; Stout et al., 1967) may have resulted from a cumulative effect over a relatively long time period, whereas the treatments of wheat seedlings were of a relatively short (2 day) period.

There are other possible explanations for our findings. A significant factor could be the rate of transport into the vacuole since it is likely that most of the TA accumulates in the vacuole (Gout et al., 1993) (e.g., K salts may promote more vacuolar accumulation than Na salts). An additional factor may be the rate of translocation of various ions to the shoot since, in wheat, the TA is formed in the shoot (Orioli and Thompson, 1990) and not in the root which is exposed to the salts. Finally, the TA content of tissues may be regulated by its rate of synthesis and degradation. However, it is not known how these metabolic processes are controlled.

Our overall conclusion from this work is that AI activity is essential for TA accumulation but does not control TA content.

ABBREVIATIONS USED

TA, *trans*-aconitate; CA, *cis*-aconitate; CTA, change in total *trans*-aconitate content; AI, aconitate isomerase activity; tris, tris(hydroxymethyl)aminomethane; mes, *N*-[morpholino]ethanesulfonic acid; gfw, grams of fresh weight; U, AI unit, nmol of TA isomerized under standard conditions (Thompson et al., 1990).

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