Effects of the Proline Analog L-Thiazolidine-4-carboxylic Acid on Proline Metabolism

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ABSTRACT

The effect of various proline analogs on proline oxidation in mitochondria isolated from etiolated barley (Hordeum vulgare) shoots was investigated. Of the analogs tested, only L-thiazolidine-4-carboxylic acid (T4C) was an effective inhibitor. T4C (1 millimolar) inhibited proline (10 millimolar) -dependent ω-uptake an average of 67%. T4C was also oxidized to some degree (12.9 nanomols oxygen per minute per milligram protein). The effect of T4C on the oxidation of other mitochondrial substrates was also tested. T4C inhibited Δ-1-pyrrolidine-5-carboxylic acid-dependent oxygen uptake slightly (13%), the oxidation of malate plus pyruvate even less (6%), and stimulated the oxidation of succinate (+11%), exogenous NADH (+19%), and citrate (+20%). Thus, inhibition by T4C in mitochondria is relatively specific to proline oxidation. T4C was found to inhibit proline dehydrogenase and not the transport of proline into the matrix.

The effect of T4C on proline metabolism in detached green barley leaves was investigated. T4C inhibited proline oxidation in turgid leaves, increasing the proline content of these leaves slightly. In wilted leaves (that are synthesizing proline rapidly), T4C inhibited proline synthesis, which resulted in a decrease in the proline content of the leaves. Δ-1-pyrrolidine-5-carboxylic acid reductase (the last enzyme in proline synthesis) was not inhibited by T4C, and thus T4C’s influence is prior to that step in the synthetic pathway. T4C had no influence on the incorporation of proline into protein.

The oxidation of proline to glutamate occurs within mitochondria (3, 7, 8), and is believed to provide energy and carbon skeletons for a number of processes. During water stress, inhibition of proline oxidation and stimulation of proline synthesis cause proline to accumulate (2, 20). Accumulated proline may lend increased survivability to stressed plants since it is a cellular osmolyte that may alleviate the effect of the stress (14, 15) and because it may serve as an energy supply for use after the stress is relieved (19). Proline also provides energy for other processes such as pollen (16) and sporal (10) germination.

The enzymes that oxidize proline (proline and P5C) dehydro-

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Abbreviations: P5C, Δ-1-pyrrolidine-5-carboxylic acid; T4C, L-thiazolidine-4-carboxylic acid (thioproline); SRM, standard reaction medium; MTT, Mes, Tes, and Tricine; PM, L-Δ-pyrrolidone methanol; A3C, L-azetidine-2-carboxylic acid; p-proline; PIP, L-pipeolic acid; 4-OH-L-PRO, 4-hydroxy-L-proline; 3,4-dehydro-ΔL-PRO, 3,4-dehydro-

Oxygen Uptake and Mitochondrial Swelling. These processes were measured simultaneously using an O2 electrode (model 53, Yellow Springs Instrument Co.) mounted in the light path of a Cary 210 spectrophotometer (Varian Instruments). The spectrophotometer was used in the single beam mode, with a period of 10 s and a band width of 0.25 nm. A full scale of 0.1 relative A230 was obtained by adjusting the gain. The O2 electrode was mounted in the top of the magnetically stirred and temperature-controlled 3 ml cuvette. The cuvette was placed within 1.5 cm of the detector window. Uptake of substances into the mitochondrial matrix results in mitochondrial swelling and a decrease in relative A230. Results are corrected for decrease in A due to

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volume changes.

O$_2$ utilization within this cuvette was measured at 26 ± 1°C in 2.2 to 2.5 ml of SRM consisting of 250 mm sucrose, 30 mm MTT (10 mm each Mes, Tes, Tricine), 1 mm KH$_2$PO$_4$, 1 mm MgCl$_2$, and 1 mg/ml BSA (pH 7.2). ADP:O ratios, RC ratios, and O$_2$ content of air-saturated water were determined according to Estabrook (9). The following mm extinction coefficients were used: $E_{340} = 6.22$ mm$^{-1}$ cm$^{-1}$ for NADH, and $E_{340} = 15.4$ mm$^{-1}$ cm$^{-1}$ for ADP (8).

Isolation and Assay of PSC Reductase from Barley. PSC reductase was isolated from etiolated barley shoots using a modification of our procedure for etiolated mung bean hypocotyls (8). The grinding medium used for this isolation was the same as that used for mitochondrial isolation from barley. The ratio of grinding medium to tissue (v/w) was 4:1. Barley PSC reductase was found to precipitate at a lower (NH$_4$)$_2$SO$_4$ concentration (30–40%) than that of mung bean (40–50%). PSC reductase activity from barley was only measurable after (NH$_4$)$_2$SO$_4$ fractionation or prolonged dialysis (6 h), indicating the presence of inhibitors within the crude cytoplasmic supernatant. This procedure resulted in a 10-fold purification and a preparation that exhibited no interfering NADH oxidation.

PSC reductase was isolated from green barley leaves (2-week-old plants) using the same procedure except that 1 mm DTT was included in the grinding medium. A 7.7-fold purification of the enzyme was obtained which showed no interfering NADH oxidation. PSC was synthesized and assayed as previously reported (8).

Experiments with Barley leaves. Barley plants were grown in soil in a growth chamber under a 16-h photoperiod (500 µE m$^{-2}$ sec$^{-1}$) at 21°C. After emergence, plants were watered daily with modified Hoagland solution (11). Fully expanded second leaves from 2-week-old barley plants were excised at the base of the leaf blade. Prior to excision, plants were removed from the growth chamber to room light (11 µE m$^{-2}$ sec$^{-1}$) to reduce transpiration rates. Leaves were weighed, then placed individually in 1 × 75-cm vials with the cut end of the leaf in 1 ml of solution. The control leaves were placed in 50 mm sucrose and 1 mM L-glutamate. T4C-treated leaves were placed in the same solution with 5 mm L-T4C. Leaves were allowed to take up the solution under room light.

Radioactive precursors were added to the cut end of the leaf in 5 µl of H$_2$O. Details of amount added, specific radioactivity, and length of pretreatment are given in the figure legends. Procedures for chromatography (2) and extraction of proline, counting, and proline determination (4) were as previously described.

RESULTS AND DISCUSSION

Mitochondrial Experiments. When etiolated barley shoots were homogenized, the resulting slurry had a yellow color. This color was removed from solution to some extent by PVP. Our experience with the isolation of PSC reductase from this tissue, has shown that proteins are inactivated by complexing with this yellow substance. Isolation of reasonably active mitochondria from etiolated barley shoots required the addition of insoluble PVP. Without addition of PVP, the oxidation of mitochondrial substrates did not show good respiratory control. RC ratios were increased an average of 23% and ADP:O ratios an average of 36% upon addition of PVP (Table I). The resulting mitochondria still appeared to be of lower quality than those obtained from corn shoots (7), but were of sufficient quality for use in investigating the inhibition of proline oxidation.

The effect of a number of proline analogs on proline-dependent O$_2$ uptake was investigated (Table II). Assays were conducted under State 3 conditions (in the presence of ADP) to maximize substrate oxidation and the resulting flow of electrons through the electron transport chain. Under these optimum conditions, the various analogs would have their greatest influence on the oxidation rate. Of those tested, only T4C was particularly effective. T4C inhibited proline-dependent O$_2$ uptake an average of 67% when used at a concentration of 1 mm. Other experiments have shown that a concentration of only 0.2 mm still resulted in over 50% inhibition. Addition of PM, A2C, D-PRO, or PIP resulted in little inhibition. Addition of 4-OH-L-PRO or 3,4-
dehydro-DL-PRO resulted in a stimulation of the O₂ uptake rate. Results similar to the above were obtained with corn mitochondria.

Because of the stimulation of O₂ uptake (by 3,4-dehydroproline in particular), we wanted to determine if any of these analogs would support O₂ uptake themselves (Table III). Using analog concentrations of 10 mM, three were found to support O₂ uptake. Oxidation of 10 mM 3,4-dehydro-DL-proline occurred at the same rate as oxidation of 10 mM L-proline. T4C inhibited the oxidation of 3,4-dehydroproline as well as it did that of proline. Surprisingly, T4C was found to support a reasonable rate of O₂ uptake. Thus, its inhibitory effect on proline oxidation may be even greater than indicated in Table II. A low rate of O₂ uptake was also obtained when 4-hydroxy-L-proline was added. PM, A2C, D-PRO, and PIP did not support any measurable O₂ uptake.

By following mitochondrial swelling at the same time as measuring O₂ uptake, we were able to roughly correlate the amount of substrate-dependent O₂ uptake to the degree of swelling (Table III). Since swelling indicates the entry of these substrates into the mitochondrial matrix (where oxidation via proline dehydrogenase occurs), it is evident that the degree of oxidation of these substrates could depend upon their transport or permeability rate in addition to their degree of reactivity with the dehydrogenase.

This result raises the possibility that T4C could inhibit proline-dependent O₂ uptake either by reducing the transport or permeability rate, or by interacting with the dehydrogenase. When T4C was added to mitochondria oxidizing proline, a rapid reduction in the O₂ uptake rate was observed. No sufficient correlating decrease in Δ⁡m was found (data not shown). If transport was inhibited, the mitochondrion would shrink due to depletion of the substrate within the matrix. This depletion of substrate would effect the inhibition of O₂ uptake. Thus, the kinetics of T4C inhibition show that T4C is a proline dehydrogenase inhibitor.

If T4C is to be useful in investigating mitochondrial proline oxidation, it must be relatively specific. To check this, we determined the effect of T4C on the oxidation of various mitochondrial substrates (Table IV). PSC-dependent O₂ uptake was inhibited slightly, and the oxidation of malate plus pyruvate even less. Succinate, exogenous NADH, and citrate-dependent O₂ uptake were all stimulated slightly by T4C. Thus, T4C's inhibitory effect is fairly specific (at least for the substrates tested). Results similar to the above were obtained with corn mitochondria.

Experiments with Green Leaves. We then investigated the effect of T4C on proline metabolism in green leaf tissue. The effect of T4C on proline oxidation in excised barley leaves is shown in Figure 1. The appearance of ^1⁴C into oxidized products

Table III. Oxygen Uptake and Mitochondrial Swelling by Various Proline Analogs

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Oxygen Uptake</th>
<th>Relative Swelling</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>nats/min-mg protein</td>
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<tr>
<td>L-PRO</td>
<td>67.0</td>
<td>+++++++</td>
</tr>
<tr>
<td>3,4-Dehydro-DL-PRO</td>
<td>68.0</td>
<td>+++++++</td>
</tr>
<tr>
<td>L-T4C</td>
<td>12.9</td>
<td>++</td>
</tr>
<tr>
<td>4-OH-L-PRO</td>
<td>6.47</td>
<td>+</td>
</tr>
<tr>
<td>L-PM</td>
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<td></td>
</tr>
<tr>
<td>L-A2C</td>
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<td></td>
</tr>
<tr>
<td>D-PRO</td>
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<tr>
<td>L-PIP</td>
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</table>

Table IV. Effect of T4C on the Oxidation of Various Mitochondrial Substrates

Assays were conducted in 2.2 to 2.5 ml of SRM containing 2.5 mM ADP. Mitochondrial protein averaged 0.94 mg/assay. The T4C concentration used was 1 mM. Results are the mean of three separate experiments.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control Rate</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nats/min-mg protein</td>
<td>%</td>
</tr>
<tr>
<td>10 mM L-proline</td>
<td>51.1</td>
<td>69</td>
</tr>
<tr>
<td>10 mM DL-PSC</td>
<td>39.9</td>
<td>13</td>
</tr>
<tr>
<td>10 mM L-malate and pyruvate</td>
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<tr>
<td>10 mM succinate</td>
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<td>+11</td>
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<tr>
<td>1 mM NADH</td>
<td>138</td>
<td>+19</td>
</tr>
<tr>
<td>10 mM citrate</td>
<td>43.1</td>
<td>+20</td>
</tr>
</tbody>
</table>

FIG. 1. Radioactivity (from ^1⁴C-proline) recovered in oxidized products in control and T4C-treated turgid leaves. After excision, control leaves were placed in 50 mM sucrose and 1 mM L-glutamate. T4C treated leaves were placed in the same solution containing 5 mM L-T4C. After a 10-h incubation period, 5 μl of L-[^1⁴C]-proline (600,000 dpm, 1.2 × 10⁹ dpm/μmol) were added to each leaf (0 time). Each value is the mean of three replicate samples.

FIG. 2. Radioactivity (from L-[^1⁴C]-proline) recovered in protein-proline from leaves described in Figure 1. (20) of proline was markedly inhibited by T4C. The specific radioactivity of proline throughout the experiment was similar in the presence and absence of T4C. Thus, the slopes of the lines can be compared directly to estimate the relative oxidation rates.
Fig. 3. Radioactivity (from L-[14C]glutamate) recovered in proline from control and T4C-treated wilted leaves. After excision, the control leaves were placed in 50 mM sucrose and 1 mM L-glutamate, and T4C-treated leaves in the same solution containing 5 mM L-T4C. After a 10-h incubation period, the leaves were wilted and 5 μL of L-[U-14C]glutamate (670,000 dpm, >200 μCi/μmol) were added to each leaf (0 time). Each value is the mean of three replicate samples.

of the two treatments. The calculated rates, using the slopes from 2 to 5 h and the specific radioactivity (20), are 0.30 and 0.09 μmol/h·g fresh weight for the control and T4C-treated leaves, respectively. These values indicate an approximate 70% inhibition of proline oxidation by T4C. However, since the proline levels were somewhat higher in the T4C-treated leaves compared to the controls (2.5 versus 2.0 μmol/g fresh weight, the degree of inhibition would be greater (20). Thus, T4C is as effective an inhibitor of proline oxidation in intact leaves as it is in isolated mitochondria. The rate of 0.3 μmol/h·g fresh weight for proline oxidation is higher than values previously reported for barley leaves, because of the higher proline levels present.

The effect of T4C on the incorporation of [14C]proline into protein is shown in Figure 2. There was no difference in the rate of incorporation in the two treatments. As stated above, since the specific radioactivity was the same in the two treatments, there was no effect of T4C on protein synthesis as measured by the incorporation of [14C]proline. Proline levels do not affect the rate of protein synthesis in leaves (20).

The effect of T4C on proline synthesis from glutamate is shown in Figure 3. These leaves were wilted to induce rapid synthesis of proline. Less conversion of [14C]glutamate to proline was observed in the T4C-treated leaves than in the controls. This inhibition could have resulted from inhibition of P5C reductase, since T4C has been reported to inhibit this enzyme (13). However, o-aminobenzaldehyde was included in the alcohol used to extract these leaves, and no 14C was recovered in the P5C-o-aminobenzaldehyde complex on the chromatograms. Thus, P5C did not accumulate as would be expected if P5C reductase was inhibited.

Experiments with P5C reductase from several sources further showed that the effect of T4C on proline synthesis is not via an effect on the reductase. P5C reductase isolated from green barley leaves had a pH optimum near 6.8 and was insensitive to T4C over a broad pH range (Fig. 4A). Since this enzyme preparation was not very active, we also isolated P5C reductase from etiolated barley shoots (Fig. 4B). The enzyme from etiolated shoots was 1.6-fold more active and was insensitive to T4C. It had the same pH optimum but higher activity from 8.0 to 8.8 as compared to green leaf P5C reductase. Our previous experience with P5C reductase from etiolated mung bean shoots had indicated a pH optimum of near pH 8.0. Thus, we ran a comparative analysis on this enzyme and found that it too was unaffected by T4C (Fig. 4C).

The mung bean P5C reductase pH curve had a shoulder between pH 6.0 and 6.4 that may correspond to the optimal activity in etiolated barley shoots. In contrast, the activity from etiolated barley shoots had a shoulder of activity that may correspond to optimum activity from mung beans. The significance of these differences in pH optima is not understood, but it has been noted before in other tissues (17). It is possible that two P5C reductases are present in plants since two P5C dehydrogenases have been found in plant mitochondria (with one oxidizing P5C derived from proline and the other oxidizing P5C...
from ornithine [8]). P5C reductase from all three sources (assayed at their respective pH optima) preferred NADH over NADPH by a factor of at least 3-fold.

Since P5C reductase is not inhibited by T4C, the effect of T4C occurs earlier in the proline biosynthetic pathway. We attempted to measure the concerted activities of glutamyl kinase, \( \gamma \)-glutamylphosphate reductase, and P5C reductase in our P5C reductase preparations and in 0 to 70\% (NH\(_4\))\( \text{SO}_4 \) fractions of the cytoplasm of these tissues. The same conditions were used as for measuring P5C reductase activity except that 10 mM L-glutamate, 1 mM ATP, and 1 mM Mg\( \text{Cl}_2 \) were added rather than P5C. No oxidation of either NADH or NADPH occurred over the pH range. Since we could not measure activity of the first two enzymes of proline synthesis, we are left speculating that T4C (due to its structural similarity to proline) may be inhibiting synthesis through feedback inhibition of glutamyl kinase.

We also checked for ornithine transaminase activity in these enzyme preparations because this activity results in P5C formation. Using the same reaction conditions as for P5C reductase (except that 10 mM L-ornithine and 10 mM \( \alpha \)-ketoglutarate were added rather than P5C), we tried to measure NAD(P)H oxidation resulting from the coupled reaction of ornithine transaminase and P5C reductase. No activity was measurable over the pH range in any of the enzyme preparations.

The effect of T4C on proline content in wilted and turgid leaves is shown in Figure 5. In turgid leaves where proline oxidation is uninhibited and proline synthesis is relatively slow, addition of T4C caused a small increase in proline levels. Thus, inhibition of proline oxidation (Fig. 1), although essential for proline accumulation to occur, is not sufficient in itself to cause accumulation.

In wilted leaves that are rapidly synthesizing proline due to loss of normal feedback inhibition (1), addition of T4C resulted in less proline accumulation. Since proline oxidation is already inhibited to a considerable extent in wilted leaves (20), the effect of T4C would be primarily on proline synthesis (Fig. 3). This supports previous findings which have shown that accumulating proline arises primarily from stimulated synthesis (2).

In conclusion, we have found an effective and relatively specific inhibitor of the mitochondrial enzyme proline dehydrogenase. It will be most useful for proline transport and oxidation research in isolated mitochondria, but will also be valuable for investigation of proline oxidation phenomena such as pollen and spore germination. In experiments with intact tissues, it should be kept in mind that T4C inhibits both proline synthesis and oxidation, thus limiting its usefulness when both processes are functioning.

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