Betaine Accumulation and Betaine-Aldehyde Dehydrogenase in Spinach Leaves

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ABSTRACT

Spinach leaf discs accumulated betaine when exposed to a mannitol solution of -20 bars. The accumulation was 12 micromoles per gram original fresh weight in a 24-hour period.

Betaine-aldehyde dehydrogenase (EC 1.2.1.8) was assayed in various subcellular fractions prepared from spinach leaves, and it was found only in the soluble fraction. This cytosolic enzyme was purified 175-fold, and its properties were studied. The enzyme was relatively specific for betaine aldehyde as the substrate with an apparent $K_m$ value of $2.08 \times 10^{-4}$ molar. It also exerted activity on other aldehyde analogs tested, but with lower $V_{max}$ and higher $K_m$ values. The enzyme was relatively specific for nicotineamide adenine dinucleotide as the coenzyme, having an apparent $K_m$ value of $9.46 \times 10^{-4}$ molar; lower activities were observed when nicotinamide adenine dinucleotide phosphate or 3-acetyl pyridine adenine dinucleotide were tested as electron acceptors. The activity was enhanced by dihydrothreitol and inhibited by $p$-chloromercuribenzoate, and the inhibition by $p$-chloromercuribenzoate was partially reversed by the subsequent addition of dihydrothreitol. The activity was inhibited by high concentrations of NaCl and, to a lesser extent, proline. The equilibrium of the enzymic reaction was strongly in favor of betaine formation.

The in vitro activity of the enzyme under optimal assay conditions was high enough to account for the amount of betaine accumulated under water stress conditions. The enzyme activity was the same in unstressed leaves and in leaves that had been water-stressed for 24 hours.

Based on the studies with mammalian systems (6, 15, 20) and on the in vivo feeding of radioactive precursors to green leaves (1, 5, 9, 10), it is known that betaine is synthesized successively from glycine, serine, ethanolamine, monomethylxantholamine, dimethylxantholamine, choline, and betaine aldehyde.

The current paper describes the partial purification and properties of betaine-aldehyde dehydrogenase (EC 1.2.1.8) from spinach leaves. This enzyme catalyzes the final reaction in the metabolic pathway of betaine synthesis.

MATERIALS AND METHODS

Plant Materials. Spinach (Spinacia oleracea L.) leaves were obtained from a local market. For water stress studies, leaf discs 1.7 cm in diameter were cut with a cork borer and allowed to float on mannitol solutions of different concentrations in Petri dishes. The Petri dishes were placed in an environmental chamber with continuous illumination under white fluorescent lamps (intensity, $9.6 \times 10^5$ erg cm$^{-2}$ s$^{-1}$) at 27°C. At 6-h intervals, the discs were removed, blotted dry, frozen in liquid $N_2$, and stored in a freezer until assayed.

Enzyme Preparation. All operations were performed at 4°C. The leaves were first chopped into small pieces with an onion chopper in a grinding medium (1 g/2 ml) containing 0.15 M Tricine-NaOH buffer (pH 8.0), 2 mM DTT, and 0.6 M sucrose; and subsequently ground gently with a mortar and pestle. This procedure did minimal damage to the organelles (11). The homogenate was filtered through four layers of cheesecloth and centrifuged at 1,000 rpm for 10 min. The supernatant fraction was then centrifuged at 10,000 rpm for 30 min, and the resulting supernatant fraction was used for enzyme purification. The supernatant fraction from 200 g of leaves was brought to 60% (NH$_4$)$_2$SO$_4$ (w/v, 36.1 g in 100 ml) with solid (NH$_4$)$_2$SO$_4$ and centrifuged at 10,000 g for 10 min. The supernatant was adjusted to 70% (NH$_4$)$_2$SO$_4$ and recentrifuged at 10,000 g for 30 min. The precipitate was resuspended in 22 ml of 10 mM K-phosphate (pH 7.5), containing 10 mM $\beta$-mercaptoethanol and 10% (v/v) glycerol. It was applied to a Sephadex G-200 column (3.5 x 8.0 cm) equilibrated with the same buffer. The proteins were eluted with the equilibrating buffer, and the fractions with enzyme activity were pooled. The pooled fractions were applied to a hydroxyapatite column (1.0 x 16 cm) and eluted with 140 ml of a linear gradient of 10 to 140 mM K-phosphate (pH 7.5), containing 10 mM $\beta$-mercaptoethanol and 10% glycerol. The fractions of peak enzyme activity were pooled.

Assays. Betaine was assayed by the method of Pearce et al. (14). Protein was determined by the Bio-Rad assay with BSA (fraction V) as the standard. Betaine-aldehyde dehydrogenase activity was assayed by either a fluorometric or a spectrophotometric method. In both assay systems, the final 3-ml reaction mixture contained 0.1 M K-phosphate (pH 8.5), 0.5 mM NAD, 5 mM DTT, 0.5 mM betaine aldehyde (Sigma), and an enzyme fraction. The reaction was initiated with the addition of substrate. Fluorescence mea-

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measurements were made with a Turner model 111 fluorometer employing excitation filter No. 7-60 (360 nm maximum) and emission filter No. 2A-12 (>510 nm) attached to a Hewlett-Packard X-Y recorder model 7034A.

RESULTS

Accumulation of Betaine by Spinach Leaf Discs under Water Stress. The spinach leaves purchased from a local store contained a large amount of betaine (Fig. 1). Presumably, this amount depended on the previous growth, shipping, and storage conditions of the plants. High (162 µmol/g dry weight) and low (23 µmol/g dry weight) quantities of betaine have been recorded for spinach (18). When the leaf discs were floated on a solution of mannitol at −20 bars, they accumulated additional betaine of approximately 12 µmol/g fresh weight in 24 h. After the leaf discs had floated on mannitol solution of different concentrations for 24 h, only those discs floated at −20 bars (lowest osmotic potential tested) accumulated betaine.

Subcellular Localization of Betaine-aldehyde Dehydrogenase. In a differential centrifugation of spinach leaf extract to yield various subcellular fractions, betaine-aldehyde dehydrogenase was recovered in the soluble fraction (Table I). From previous (11, 13) and current experiments (data not shown), it was found that most of the chloroplasts, mitochondria, and peroxisomes sedimented at 1,000g and 10,000g, and some of the ER sedimented at 10,000g. Inasmuch as betaine-aldehyde dehydrogenase was not associated with these pellet fractions, it can be assumed to be a cytosolic enzyme.

Purification and Properties of Betaine-aldehyde Dehydrogenase. The supernatant fraction obtained after centrifugation of the homogenate at 10,000g for 30 min was used to purify betaine-aldehyde dehydrogenase. After fractionation with successive ammonium sulfate precipitation, Sephadex G-200 chromatography, and hydroxyapatite chromatography, the enzyme was purified 175-fold (Table II). This partially purified enzyme preparation was used to study the catalytic properties of the enzyme.

The enzyme showed maximal activity at pH 8.5 (Fig. 2). It was relatively specific for betaine aldehyde as the substrate, with an apparent $K_m$ value of 2.08 × 10⁻⁴ M (Fig. 3). Of the various aldehyde analogs tested as potential substrates, betaine aldehyde gave the highest $V_{\text{max}}$ value (355 nmol/min·mg protein) as well as the lowest $K_m$ value. D.L-5-Glyceraldehyde exerted a $V_{\text{max}}$ value of 236 nmol/min·mg protein and a $K_m$ value of 4.76 × 10⁻³ M.

Table II. Purification of Betaine-Aldehyde Dehydrogenase from Spinach Leaves

Two hundred g of leaves were used. The initial homogenization was performed gently to preserve the integrity of organelles, and, therefore, the total activity was lower than that from a complete extraction (36 nmol/min·g).

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000g</td>
<td>nmol/min</td>
<td>mg</td>
<td>nmol/min·mg</td>
<td>%</td>
<td>fold</td>
</tr>
<tr>
<td>Supernatant</td>
<td>1,298</td>
<td>705</td>
<td>1.8</td>
<td>100</td>
<td>1.7</td>
</tr>
<tr>
<td>Ammonium sulfate (60 to 70% saturated)</td>
<td>402</td>
<td>55.4</td>
<td>7.3</td>
<td>31</td>
<td>6.5</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>473</td>
<td>12.5</td>
<td>37.8</td>
<td>36</td>
<td>34.2</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>257</td>
<td>1.3</td>
<td>193.8</td>
<td>20</td>
<td>175.4</td>
</tr>
</tbody>
</table>

Fig. 1. Amount of betaine accumulated by spinach leaf discs. A, leaf discs were floated on mannitol solution of −20 bars for various lengths of time; B, leaf discs were floated on mannitol solution of indicated water potential for 24 h.

Table I. Distribution of Betaine-Aldehyde Dehydrogenase in Subcellular Fractions of Spinach Leaves

Twenty g of spinach leaves were used.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>186</td>
<td>214</td>
<td>0.87</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>1,000g Pellet</td>
<td>0</td>
<td>45</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10,000g Pellet</td>
<td>0</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10,000g Supernatant</td>
<td>223</td>
<td>154</td>
<td>1.45</td>
<td>120</td>
<td>1.67</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of pH on activity of partially purified betaine aldehyde dehydrogenase. One-hundred per cent activity is equivalent to 0.53 nmol/min·min. —, K-phosphate buffer (0.1 M); —, Na-borate buffer (0.1 M).

Fig. 3. Double reciprocal plot of activity of partially purified betaine aldehyde dehydrogenase as a function of concentration of betaine aldehyde. Protein (2.34 µg) was used in each assay.
Table III. Effect of DTT and PCMB on the Activity of Partially Purified Betaine-Aldehyde Dehydrogenase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>5 mM DTT (10 min)</td>
<td>140</td>
</tr>
<tr>
<td>5 mM DTT (30 min)</td>
<td>179</td>
</tr>
<tr>
<td>0.1 mM PCMB (10 min)</td>
<td>0</td>
</tr>
<tr>
<td>5 mM DTT (30 min), then 0.1 mM PCMB (30 min)</td>
<td>99</td>
</tr>
<tr>
<td>0.1 mM PCMB (10 min), then 5 mM DTT (30 min)</td>
<td>21</td>
</tr>
</tbody>
</table>

and acetaldehyde and glycolaldehyde, at concentrations between 0.1 and 100 mM, could not serve as substrates. The enzyme was relatively specific for NAD as the electron acceptor with a low \( K_m \) value of 9.46 \( \times 10^{-6} \) M (Fig. 4); it had a lower activity toward NADP, the \( V_{max} \) value being one-fourth of that toward NAD and the \( K_m \) value being 6.35 \( \times 10^{-4} \) M. 3-Acetyl-pyridine adenine dinucleotide could also be used as a coenzyme, but at 0.5 mM, it yielded only 39% of the activity obtained with the same concentration of NAD.

The enzyme activity was enhanced by DTT and inhibited strongly by PCMB\(^4\) (Table III). Inhibition by PCMB was partially reversed by the subsequent addition of DTT, and preincubation of the enzyme with DTT could partially protect the enzyme from subsequent inhibition by PCMB. Therefore, one or more sulfhydryl group in the enzyme is essential for activity.

The enzyme activity was inhibited by NaCl at high concentrations; 50% of the activity was lost with 0.5 mM NaCl (Fig. 5). Proline at high concentration was also inhibitory, but the inhibition was less than that with NaCl.

The reaction equilibrium was studied by measuring spectrophotometrically NADH produced or consumed. The reverse reaction, when assayed with the same concentration of 0.5 mM NADH and 0.5 mM betaine, was not detectable, even though our assay system could detect less than 1% of the forward activity. Using betaine at concentrations of 10, 50, and 100 mM, we still were unable to detect the reverse reaction. When the forward reaction was assayed using 0.05 mM NAD and 1.0 mM betaine aldehyde, most of the NAD was converted to NADH (using 2.6 cm\(^2\)/\( \mu \)mol as the extinction coefficient of NADH). To calculate the equilibrium constant of the reaction, the exact amount of the trace NAD left in the forward reaction has to be known. However, we do not know the exact percentage of active NAD in the Sigma grade III and IV that we used. Nevertheless, the experimental results indicate that

\(^4\) Abbreviation: PCMB, \( p \)-chloromercuribenzoate.

Fig. 4. Double reciprocal plot of activity of partially purified betaine aldehyde dehydrogenase as a function of concentration of NAD. Protein (2.34 \( \mu \)g) was used in each assay.

Fig. 5. Effect of proline and NaCl on activity of partially purified betaine aldehyde dehydrogenase. One-hundred percent activity is equivalent to 0.53 nmol/min.

the equilibrium of the reaction is strongly in favor of betaine formation.

Enzyme Activity Before and After Water Stress. After a thorough homogenization of spinach leaves, 36 nmol/min·g fresh weight of betaine-aldehyde dehydrogenase activity was obtained. This activity is sufficient to account for the observed rate of betaine accumulation in vivo (Fig. 1). We were able to extract 86% of the above activity from leaf discs that had been subjected to water stress treatment for 24 h at -20 bars. Therefore, the enzyme activity did not go through a substantial alteration during the water stress period.

DISCUSSION

Although betaine synthesis and accumulation is very important in the physiology of many higher plants during water, salt, or cold stress (4, 7, 9, 18, 21), the enzymes of betaine metabolism have never before been studied in detail. The enzymes of betaine metabolism in mammalian tissues have been studied (6, 15, 20). Whether or not higher plants also possess similar enzymes is unknown. There are a few fundamental differences in the betaine metabolism between mammalian and plant tissues. Mammalian tissues do not accumulate betaine but actively utilize it as a methyl group donor. In higher plants, the stressed tissues accumulate betaine but do not utilize it actively after the stress, and, thus far, little evidence has been presented showing its activity as a methyl group donor.

Betaine-aldehyde dehydrogenase in the current study appears to be the enzyme responsible for betaine synthesis in spinach leaves. It is relatively specific for betaine aldehyde as the substrate. Its activity is high enough to account for the amount of betaine synthesized during water stress, and, furthermore, it remains unchanged during water stress. It is of physiological significance that the reaction which the enzyme catalyzes is strongly in favor of betaine formation. The accumulated betaine in red beet (21) and \textit{Suaeda maritima} (8) has been shown to localize not in the vacuole but in the cytoplasm. Inasmuch as the enzyme is found in the cytosol, only an equilibrium that is strongly in favor of betaine formation allows such an accumulation of betaine within the same cellular compartment. The finding that the enzyme activity is not enhanced during water stress and that its \textit{in vitro} activity is not promoted by NaCl or proline suggests that the cellular regulation of betaine accumulation may not occur at the conversion of betaine aldehyde to betaine. However, the possibility of having control by alteration of subcellular compartmentation of the enzyme, substrate, and coenzyme during water stress cannot be ruled
out. Results of in vivo feeding experiments are in agreement with a lack of control at the conversion of betaine to betaine aldehyde (10).

The catalytic properties of the spinach enzyme are quite similar to those of the betaine-aldehyde dehydrogenase derived from rat liver (6, 15, 20). In rat liver, most of the activity is present in the cytosol. A small percentage (3–7%) of the activity is localized in the mitochondria (20), but this activity belongs to a nonspecific aldehyde dehydrogenase (6). In spinach leaves, we found the enzyme to be in the cytosol, and we could not detect any activity in the particulate fractions. Even if a few percentage of the activity were present in the mitochondria of spinach leaves, the mitochondrial activity would not be enough to account for the amount of betaine accumulated during water stress. Our calculation is based on the total enzyme activity of 36 nmol/min·g fresh weight at 24 C and the accumulation of 17 nmol/min·g fresh weight of betaine at 27 C during the 12- to 24-h period of water stress (Fig. 1).

It has been proposed that betaine and proline are cytoplasmic osmotica that are synthesized by plant tissues in response to water/salt stress. Under water/salt stress condition, the betaine content is several-fold higher than the proline content in the tissues. However, the rates of accumulation of proline (12) and betaine (Fig. 1) appear to be approximately the same in spinach. Whereas the accumulated proline is catabolized rapidly after the stress, the accumulated betaine remains unmetabolized in the tissues (3, 9). It is possible that plants accumulate proline to combat the current stress only and betaine to combat the current stress as well as to acclimate the tissue to future stress. If so, betaine can be a very useful osmotica, especially since, at high concentrations, it is relatively nontoxic to the cells. The drawback is that a large proportion of the valuable cellular nitrogen is locked into betaine.

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