Betaine Aldehyde Oxidation by Spinach Chloroplasts

PIERRE WIEGEL, ELIZABETH A. WERETILNYK, AND ANDREW D. HANSON
MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Chenopods synthesize betaine by a two-step oxidation of choline: choline \(\rightarrow\) betaine aldehyde \(\rightarrow\) betaine. Both oxidation reactions are carried out by isolated spinach (Spinacia oleracea L.) chloroplasts in darkness and are promoted by light. The mechanism of betaine aldehyde oxidation was investigated with subcellular fractions from spinach leaf protoplasts. The chloroplast stromal fraction contained a specific pyridine nucleotide-dependent betaine aldehyde dehydrogenase (about 150 to 250 nanomoles per milligram chlorophyll per hour) which migrated as one isozyme on native polyacrylamide gels stained for enzyme activity. The cytosol fraction contained a minor isozyme of betaine aldehyde dehydrogenase. Leaves of pea (Pisum sativum L.), a species that lacks betaine, had no betaine aldehyde dehydrogenase isozymes. The specific activity of betaine aldehyde dehydrogenase rose three-fold in spinach plants grown at 300 millimolar NaCl; both isozymes contributed to the increase. Stimulation of betaine aldehyde oxidation in illuminated spinach chloroplasts was due to a thylakoid activity which was sensitive to catalase; this activity occurred in pea as well as spinach, and so appears to be artificial. We conclude that in vivo, betaine aldehyde is oxidized in both darkness and light by the dehydrogenase isozymes, although some flux via a light-dependent, \(H_2O_2\)-mediated reaction cannot be ruled out.

Betaine (glycinebetaine) accumulates in response to salinization or to water deficit in chenopods, grasses, and in other angiosperm families (8) as well as in a number of prokaryotes (11, 14). For higher plants, Wyn Jones et al. (30) have proposed that betaine is localized mainly in the cytoplasm, where it acts as a nontoxic osmoticum, allowing osmotic adjustment to occur without perturbing metabolic functions. Much evidence (7) now supports this proposal, which accords betaine synthesis a major role in adaptation to osmotic stress. In-vivo radiotracer studies (8) show that betaine is synthesized in leaves from a two-step oxidation of choline:

\[
\text{Choline } \rightarrow \text{betaine aldehyde } \rightarrow \text{betaine}
\]

Little is known about the nature of these reactions in plants or about the enzymes involved. However, the enzymology of choline oxidation is quite well known for mammalian liver, in which both steps are mitochondrial (10, 29), and for certain microorganisms (15–17). In these nonplant systems the choline \(\rightarrow\) betaine aldehyde step is catalyzed by a flavoprotein dehydrogenase or oxidase, the betaine aldehyde \(\rightarrow\) betaine step by a specific pyridine nucleotide dehydrogenase. In the case of liver, some betaine aldehyde oxidation may also occur via cytosolic dehydrogenase(s) (29).

We recently showed that both steps in choline oxidation are chloroplastic in spinach, that they are light-promoted, and that the effect of light is sensitive to DCMU (9). On the other hand, Pan et al. (19) reported that the cytosolic fraction from spinach leaves contained NAD-dehydrogenase activity specific for betaine aldehyde, and that the chloroplastic fraction lacked this activity. Therefore, in this work we examined (a) the mechanisms by which spinach chloroplasts oxidize betaine aldehyde in darkness and light, and (b) the subcell distribution, specificity, and isozyme composition of spinach betaine aldehyde dehydrogenase.

MATERIALS AND METHODS

Plant Material. All spinach plants (cv Savoy Hybrid 612) were grown in 8-h d in the growth chamber conditions given previously (9). Spinach plants for protoplast preparation were grown in flats of vermiculite and watered with half-strength Hoagland solution. Spinach plants for salinization experiments were grown individually in plastic 350-ml pots of a 1:1 mix of vermiculite and gravel. Salinization began when plants had two true leaves (about 2 weeks after emergence) with 50 mm NaCl in half-strength Hoagland solution (100 ml/d per pot). This NaCl level was continued for 3 d, and thereafter raised in 50 mm steps every 3 d until the desired final level was reached. Plants were used for experiments after at least 7 d at the desired final NaCl level. In experiments with a range of final NaCl levels, salinization of all treatments started at the same time, and plants were held at the various intermediate NaCl levels until the highest NaCl treatment was completed, at which time all treatments were harvested. Pea (Argentum mutant) plants for protoplast preparation were grown as described previously (9). Pea plants (cv Little Marvel) for isozyme tests were grown in trays of vermiculite in the chamber used for salinization.

Protoplast Preparation and Fractionation. Spinach and pea leaf protoplasts were obtained using sterile procedures (9), and checked for photosynthetic activity in an O\(_2\) electrode at 25°C and saturating light. The electrode medium contained 50 mm Hepes/KOH, 1 mm CaCl\(_2\), 0.5 m sorbitol, 1 mg/ml BSA, 10 mm NaHCO\(_3\), adjusted to pH 7.8; rates of O\(_2\) evolution were 30 ± 3, and 56 ± 7 \(\mu\)mol/mg Chl-h for spinach and pea, respectively (± SE). Microbial contamination was monitored (9); preparations with >2 colony forming units/10\(^4\) protoplasts were not used.

All operations were at 0 to 4°C except for high-speed centrifugations which were in a Beckman Airfuge (18° angle rotor, 27,000 g) at room temperature. Organelles were released from protoplasts by resuspending intact protoplasts at about 1 mg Chl/ml in lysis medium (50 mm Hepes/KOH [pH 7.6], 0.45 m sorbitol, 1 mm Na\(_2\)EDTA, 2 mg/ml BSA, 10 \(\mu\)g/ml chloramphenicol) and drawing the suspension three to four times in and out of a 1-ml syringe closed with one layer of 15-\(\mu\)m nylon mesh. Lysates were fractionated by differential centrifugation or by a

1 Funded by Department of Energy Contract DE-AC02-76ERO-1338, and by grants from CIBA-GEIGY Corporation and the Michigan Sugar Company.

2 Permanent address: Laboratoire de Biologie et Physiologie Végétales, Université de Rennes I, Rennes, France. P. W. was supported by a fellowship from the French Ministry of Foreign Affairs.
Percoll step procedure (9). Particulate fractions were washed as specified in the text and resuspended in lysis medium. Chloroplast intactness was routinely evaluated by phase-contrast microscopy or ferriyedan reduction (13). For osmotic lysis, fractions were resuspended in lysis medium without sorbitol; when isozymes were studied, the BSA level in this medium was lowered to 1 mg/ml. Fractions were dialyzed with Amicon Centrincon-30 microconcentrators, reducing the volume about 10-fold and diluting with lysis medium minus sorbitol, three times. Betaine aldehyde oxidizing activity did not pass the membrane. Alternatively, fractions were treated with Sephadex G-25 as described below.

Chloroplasts and Extracts from Leaves. Operations were at 0 to 4°C. Chloroplasts were isolated directly from spinach leaves by a modification of standard methods (27). Deveined leaf tissue (10 g) was cut into 2-mm strips and ground for 3 s in a Polytron blender in 40 ml semifrozen grinding medium containing Mes/NaOH 30 mM, sorbitol 0.33 M, MgCl₂; 5 mM, Na₂EDTA 2 mM, 1 mg/ml BSA, and 10 mM ascorbate, adjusted to pH 6.5. The sorbitol level was raised to 0.59 M for salinization (200 mM NaCl plants). The homogenate was squeezed through six layers of cheesecloth and filtered through a layer of cotton wool between two layers of cheesecloth; 10-ml portions were then layered onto 5 ml cushions of 40% Percoll medium containing the following buffer: Hepes/KOH 50 mM (pH 7.6), sorbitol 0.33 or 0.59 M, 5 mM DTT, 1 mM EDTA, and 1 mg/ml BSA. After centrifuging at 3,000g for 2.5 min, the pellet, containing >90% intact chloroplasts, was lysed by resuspending in the above buffer without sorbitol. The lysate was centrifuged at 10,000g for 5 min and the supernatant was then centrifuged in an Airfuge for 10 min; the second supernatant (stromal fraction) was treated with Sephadex G-25 as described below.

Whole-leaf extracts were prepared by thoroughly grinding leaf tissue in Hepes/KOH 50 mM (pH 7.6 or 8.0), Na₂EDTA 1 mM, DTT 5 mM, BSA 1 or 2 mg/ml (extraction medium, 2 mg/l fresh weight) using a pestle and mortar with sand. The homogenate was squeezed through six layers of cheesecloth and centrifuged at 10,000g for 10 min. The pH of the supernatant was adjusted to 7.6 or 8.0 and samples (≤0.2 ml) were centrifuged through 1.1-ml Sephadex G-25 columns, equilibrated with 50 mM Hepes/KOH (pH 7.6 or 8.0) containing 1 mM Na₂EDTA, to remove low mol wt compounds before enzyme assay.

Enzyme Assays. Oxidation of [¹⁴C]betaine aldehyde to betaine in darkness or red light (2000-4000 μE/m²·s) was assayed essentially as described (9). Reaction mixtures (55-66 μl) contained 27 nmoles of [¹⁴C]betaine aldehyde (0.5-3 μCi/μmol), Hepes/KOH (pH 7.6 or 8.0) and 1 mM Na₂EDTA. Other additions are given in the text. Amount of enzyme and incubation time (30 min-2 h) were adjusted so that less than one-third of the substrate was consumed. Incubation temperature was 25 to 27°C; assays were shaken at 300 (darkness) or 60 (light) cycles/min on a rotary shaker. [¹⁴C]Betaine formed was separated by ion-exchange chromatography (9) and estimated by scintillation counting; TLC and electrophoresis of representative samples verified that the labeled reaction product was betaine. Betaine aldehyde dehydrogenase was assayed spectrophotometrically in 1-ml reaction mixtures containing 50 mM Hepes/KOH (pH 7.6), 1 mM betaine aldehyde, and 1 mM NAD. GAPDH (26) and SKDH (3) were used as stromal markers, and fumarase (21) as a mitochondrial marker. Catalase and Chl were assayed as in Hanson et al. (9). Protein was determined by the method of Bradford (1) using BSA as a standard.

Results

Dark [¹⁴C]Betaine Aldehyde Oxidation in Protoplast Lysate Fractions. Figure 1A confirms and extends our earlier report (9) that most of the betaine aldehyde-oxidizing activity of spinach protoplast lysates is darkness recovered in the 500g (chloroplast) pellet and 15,000g supernatant, with less in the 15,000g (mitochondrial) pellet. Osmotic lysis released much latent activity from the 500g pellet, but little from the 15,000g pellet. Addition of a mixture of NAD and NADP modestly increased the activity of the chloroplast lysate and the supernatant. Because approximately 15% of the chloroplasts in the 500g pellet were broken, we inferred that much of the supernatant activity was probably of chloroplast origin, and so focused initially on the chloroplast activity.

First, the possibility that the latent chloroplast activity is simply a nonspecific aldehyde oxidizing system was evaluated by testing

---

3 Abbreviations: GAPDH, NADP-glyceraldehyde-3-phosphate dehydrogenase; BALDH, betaine aldehyde dehydrogenase; PCMB, p-chloromercuribenzoic acid; SKDH, shikimate dehydrogenase; SOD, superoxide dismutase.
chloroplasts of pea, a species that does not accumulate betaine (9). Pea chloroplasts showed 15-fold less latent activity than spinach (Fig. 1B). The oxidizing activity of spinach chloroplasts was then examined in more detail (Fig. 2). After differential centrifugation of chloroplast lysates, the latent activity remained in the 170,000g supernatant and, as in Figure 1, was somewhat increased by NAD. Following dialysis, the activity of the supernatant fell to a low level, but was restored by addition of either NAD or NADP; the effects of the pyridine nucleotides were not additive. In six experiments of this type, NADP always gave slightly lower activity than NAD, by an average of 14%. Table I demonstrates that NAD-dependent oxidation of [14C]betaine aldehyde by the stromal fraction is linear with time, that flavins cannot replace NAD, and that although the activity is unaffected by DTT, it is inhibited by the sulphydryl reagent PCMB. The presence of a pyridine nucleotide dependent dehydrogenase was confirmed by assaying NAD reduction; agreement between spectrophotometric and radiochemical assays was good ($r^2 = 0.99$, $n = 5$). At saturating substrate and cofactor levels (pH 7.6), the rate of NADP reduction was about 30% less than NAD reduction, confirming some preference for NAD. Spectrophotometric assays indicated a pH optimum of 7.5 to 8.0 (not shown). For subsequent work, NAD-dependent [14C]betaine aldehyde oxidation by dialyzed extracts at pH 7.6 or 8.0 was used as a measure of BALDH activity.

Evidence for Stromal and Cytosolic Betaine Aldehyde Dehydrogenases. The distribution of BALDH between chloroplast and extrachloroplastic (cytosol) fractions of protoplast lysates was compared with that of the stromal marker enzymes GAPDH and SKDH; Table II gives two typical sets of results. The distribution of BALDH between chloroplast and cytosol fractions was always similar to that of the stromal markers but in some cases there was 10 to 15% more BALDH in the cytosol fraction than expected for a stromal enzyme, suggesting that a minor cytosolic form might be present as well as the major stromal form. This was confirmed when chloroplast and cytosol fractions were separated on native PAGE and stained for BALDH activity (Fig. 3A). Stromal fractions contained one prominent band; cytosol fractions showed the stromal band and a second minor band that migrated more slowly. The stromal band in the cytosol fraction can be attributed to contamination from chloroplast breakage, because 23% of the GAPDH and 22% of the SKDH activity was recovered in the cytosol fraction in this experiment. The cytosol-specific band was always present but varied in staining intensity relative to the stromal band, consistent with the observed variation in extrachloroplastic activity (Table II).

Because of the report that BALDH is predominantly cytosolic in spinach (19), subcell localization was checked in two further ways. First, chloroplasts were purified from protoplast lysates using a Percoll cushion (Table III). Note (a) that the protoplasts were from rapidly expanding young leaves, so that plastids were not all fully differentiated, and (b) that Percoll probably discriminated in favor of mature chloroplasts because the pellet was somewhat enriched in the photosynthetic enzyme GAPDH. Despite this constraint on quantitative interpretation, the data for BALDH are clearly inconsistent with a mainly cytosolic enzyme. In the second type of experiment, chloroplasts were prepared directly from young expanded leaves using Percoll. The percent recovery of BALDH in the purified chloroplasts was similar to

---

**Table I. Some Properties of BALDH Activity from Spinach Chloroplasts**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pretreatment</th>
<th>Assay Conditions</th>
<th>[14C]Betaine Aldehyde Oxidation (nmol/mg Chl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>−NAD, 1 h</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>+Flavins*, 1 h</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>+NAD, 0.5 h</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>+NAD, 1 h</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>DTT 5 mm, 10 min</td>
<td>+NAD, 2 h</td>
<td>232</td>
</tr>
<tr>
<td></td>
<td>DTT 5 mm, 10 min</td>
<td>+NAD, 1 h</td>
<td>127</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>+NAD, 1 h</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>PCMB 0.1 mm, 10 min</td>
<td>+NAD, 1 h</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>PCMB 0.3 mm, 10 min</td>
<td>+NAD, 1 h</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* FMN 1 mm plus FAD 1 mm.  
* NAD was 2 mm.
Table II. Distribution of BALDH Activity in Relation to Stromal Marker Enzymes in Spinach Protoplast Fractions

BALDH activity was estimated as NAD-dependent $[^3]C$ betaine aldehyde oxidation in dialyzed extracts. In Experiment 1, chloroplast intactness (by phase contrast) = 64%, Chl fractionated = 1.43 mg. Experiment 2, chloroplast intactness = 71%, Chl fractionated = 0.97 mg.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stroma</td>
<td>Cytosol</td>
</tr>
<tr>
<td>GAPDH (nmol/min)</td>
<td>1540</td>
<td>644 (29)*</td>
</tr>
<tr>
<td>SKDH (nmol/min)</td>
<td>217</td>
<td>119 (35)</td>
</tr>
<tr>
<td>BALDH (nmol/h)</td>
<td>101</td>
<td>45 (31)</td>
</tr>
</tbody>
</table>

* Values in parentheses are the percentage of the total enzyme activity recovered in the cytosol fraction.

Table III. Recovery of BALDH and Marker Enzymes in Spinach Chloroplasts Purified from Protoplast Lysates with Percoll

BALDH activity was estimated as NAD-dependent $[^3]C$ betaine aldehyde oxidation in dialyzed extracts. Total protoplast extract was obtained by osmotic lysis of whole protoplasts, followed by high-speed centrifugation. The stromal fraction of Percoll-purified chloroplasts was also obtained by osmotic lysis and high-speed centrifugation. The chloroplasts purified by Percoll were 91% intact (phase-contrast) before lysis.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Total protoplast extract</th>
<th>Chloroplast fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chl (µg)</td>
<td>1127</td>
<td>168 (14.9)*</td>
</tr>
<tr>
<td>Catalase (µmol/min)</td>
<td>2184</td>
<td>7 (0.3)</td>
</tr>
<tr>
<td>Fumarase (nmol/min)</td>
<td>174</td>
<td>1.1 (0.6)</td>
</tr>
<tr>
<td>GAPDH (nmol/min)</td>
<td>1250</td>
<td>308 (24.6)</td>
</tr>
<tr>
<td>SKDH (nmol/min)</td>
<td>243</td>
<td>45 (18.4)</td>
</tr>
<tr>
<td>BALDH (nmol/h)</td>
<td>299</td>
<td>45 (15.0)</td>
</tr>
</tbody>
</table>

* Values in parentheses are the percentage of the total marker recovered in the chloroplast fraction.

Table IV. Activities of BALDH and SKDH in Spinach Leaf Extracts and Percoll-Purified Chloroplasts

Chloroplasts were prepared directly from leaves; intactness in both experiments was >90%. Sorbitol concentrations in the grinding medium were 0.33 M for unsalinized plants and 0.59 M for salinized plants. The BALDH assay medium for the stromal fraction was Hepes/KOH 50 mM (pH 7.6), Na$_2$EDTA 1 mM; that for the leaf extracts contained in addition 2.5 mM DTT and 0.5 mg/ml BSA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Marker</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young leaves</td>
<td>Chl (µg)</td>
<td>8440</td>
</tr>
<tr>
<td>0 mM NaCl</td>
<td>SKDH (nmol/min)</td>
<td>4080</td>
</tr>
<tr>
<td></td>
<td>BALDH (nmol/h)</td>
<td>3430</td>
</tr>
<tr>
<td>Mature leaves</td>
<td>Chl (µg)</td>
<td>16200</td>
</tr>
<tr>
<td>200 mM NaCl</td>
<td>SKDH (nmol/min)</td>
<td>5460</td>
</tr>
<tr>
<td></td>
<td>BALDH (nmol/h)</td>
<td>7550</td>
</tr>
</tbody>
</table>

* Values in parentheses are the percentage of the total marker recovered in the chloroplast fraction.

**Response of Betaine Aldehyde Dehydrogenases to Salinity.** Salinized spinach plants showed the expected slowed growth, lowered $\psi_0$, and increased betaine level (Fig. 4). Total BALDH activity rose about 3-fold as the NaCl concentration was increased from 0 to 300 mM. Although enzyme activity is given on a fresh weight basis in Figure 4, data expressed as specific activity show the same 3-fold increase because soluble protein levels were 9 to 11 mg/g fresh weight at all salt levels. Native PAGE of leaf extracts from unsalinized and salinized plants (Fig. 5) indicated that the stromal isozyme remained the more prominent activity after salination; although the relative staining intensity of the cytosolic isozyme showed a modest increase, this appeared too small to account for the 3-fold rise in total activity which is therefore best explained by contributions from both isozymes.

Consistent with a predominantly stromal location, the percent recovery of BALDH in Percoll-purified chloroplasts from salinized leaves was similar to that of the stromal marker SKDH (Table IV). To examine the effect of sudden salt shock, 6-week-old plants were transferred from 0 to 200 mM NaCl. Turgor was rapidly lost, and regained after about 2 d. BALDH activity did not change abruptly; it began to rise during the 1st d, and reached 3-fold the initial value 7 d after the salt treatment started (not shown).

BETaine ALDEHyDE OXIDATION BY SPINACH CHLORoplasts

**Fig. 4.** Effect of salinization on growth and solute potential (A), betaine level (B), and BALDH activity (C) in spinach shoots. Plants were 38 d old at harvest. Before enzyme assay, extracts were passed through Sephadex G-25; BALDH activity is the increase in \(^{14}C\)betaine aldehyde oxidation given by NAD. Enzyme activity values can be converted to a Chl basis by using a value of 2 mg Chl/g fresh weight for all treatments. The experiment was repeated three times, with similar results.

**Fig. 5.** Isozyme profiles of BALDH in whole leaf extracts from unsalinized and salinized (200 mM NaCl) spinach plants similar to those of Figure 4. The unsalinized track contained 3.5-fold more leaf extract (equivalent to 137 mg fresh weight) than the salinized track (equivalent to 39 mg). Both tracks were stained for enzyme activity for 45 min.

Betaine aldehyde oxidation by intact spinach chloroplast preparations is stimulated several-fold by high intensity light, and this stimulation is sensitive to DCMU (9). Table V demonstrates this light effect in spinach chloroplasts, and shows also a very similar effect in chloroplasts of pea, a plant which does not accumulate betaine. Methyl viologen enhanced the effect of light in both species, suggesting that \(H_2O_2\) might be the oxidant. Spinach and pea thylakoid preparations oxidized betaine aldehyde even more actively than chloroplasts (Fig. 6). Although spinach thylakoids were completely inactive in darkness, a few minutes of room light was sufficient to ensure measurable oxidation (Fig. 6A).

**Table V. Effects of Light and Methyl Viologen on Betaine Aldehyde Oxidation by Spinach and Pea Chloroplasts**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spinach</th>
<th>Pea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark</td>
<td>19</td>
<td>4</td>
</tr>
<tr>
<td>Dark + MV</td>
<td>24</td>
<td>ND*</td>
</tr>
<tr>
<td>Light</td>
<td>141</td>
<td>104</td>
</tr>
<tr>
<td>Light + MV</td>
<td>209</td>
<td>225</td>
</tr>
</tbody>
</table>

*Not determined.

**Fig. 6.** Time courses of the oxidation of \(^{14}C\)betaine aldehyde to betaine by washed thylakoids of spinach (A) and pea (B). (O), red light (2000-4000 \(\mu\)E/m\(^2\cdot\)s); (●), complete darkness. Assays contained 29 mg Chl. The single point (Δ) at 10 min for spinach shows the effect of room light.

Consistent with the possibility that \(H_2O_2\) is the oxidant, catalase abolished light-dependent betaine aldehyde oxidation by spinach and pea thylakoids whereas SOD was without effect (Table VI); with thylakoids, methyl viologen enhanced oxidation slightly or not at all.

**DISCUSSION**

Our results establish that spinach chloroplasts contain a pyridine nucleotide linked betaine aldehyde dehydrogenase which
accounts for most of the potential for dark oxidation of betaine aldehyde in cell extracts. The data also indicate that this enzyme is specific to the betaine pathway, since it is lacking in pea and since it does not readily attack other small aldehydes. Because our stromal dehydrogenase was similar in activity level, preference for NAD, substrate range, and pH optimum to the enzyme partially purified by Pan et al. (19) we conclude that these are one and the same enzyme. Pan et al. supposed their enzyme to be cytosolic, based on differential centrifugation of leaf homogenates prepared with a mortar and pestle. We suggest that chloroplastic breakage was massive in their experiments, and that the stromal enzyme was released.

The apparent ability of a stromal enzyme to use either NAD or NADP, with some preference for NAD, is unusual and will be interesting to study in detail with a purified preparation. In being able to use either pyridine nucleotide the stromal enzyme resembles the BALDH of Pseudomonas aeruginosa (15), and differs from the mammalian liver enzyme, which is NAD-specific (24). Because NAD/H and NADP/H pools in the chloroplast are not in equilibrium (23), an NAD-linked BALDH might serve to isolate betaine aldehyde oxidation from general control exerted by NADP/H over photosynthetic carbon reduction. Some means of freeing betaine synthesis from photosynthetic regulatory mechanisms would appear essential, especially as the water or salt stress treatments that elicit betaine accumulation reduce or completely inhibit CO₂ uptake.

The presence of a minor cytosolic (extrachloroplastic) isozyme which, like the stromal enzyme, appears to be specific for betaine aldehyde and to prefer NAD, conforms to a general pattern whereby the same reaction in different cellular compartments is catalyzed by different isozymes (5). However, the function of the cytosolic isozyme is not obvious. The chloroplast is evidently the sole site of betaine aldehyde synthesis (9), and while it is conceivable that betaine aldehyde is exported from the chloroplast to meet the cytoplasmic requirement for betaine, betaine itself appears to cross the chloroplast envelope very readily (22), obviating the need for the precursor to exit. However, were betaine aldehyde as easily lost from the chloroplast as betaine (22), some betaine aldehyde leakage might be inevitable, in which case a cytosolic isozyme could play a scavenging role. The cytosolic isozyme, like the stromal isozyme, is not likely to function as a betaine reductase, (a) because the redox potential of the betaine aldehyde/betaine system is far lower than that of NADH/NAD⁺, making the oxidation reaction essentially irreversible (19), and (b) because supplied [¹⁴C]betaine is not metabolized in vivo (8).

We take the salt induced rise in BALDH, observed also by Pan (18), as good circumstantial evidence that this activity is physiologically relevant to betaine synthesis. Although the 3-fold induction was not large compared, for example, with anaerobic induction of alcohol dehydrogenases (4), it is more than sufficient to account for the observed 6-fold increase in betaine concentration in plants whose growth was severely depressed. Because the increase in total enzyme activity upon salination could not be attributed to either isozyme alone, and because chloroplastic and cytosolic isozymes are likely to be products of separate genes (5), coordinate control of gene expression may be involved. It is possible that coordinate regulation extends also to choline oxidation, the first step of the betaine pathway, and the probable flux-generating reaction. However, since the mechanism of choline oxidation in chloroplasts is at present unknown it may be that BALDH is itself the flux-generating step and is the only stress-regulated enzyme.

The light-dependent betaine aldehyde oxidizing activity of chloroplasts and thylakoids has several features indicative of a nonphysiological process: (a) It is as active in pea as in spinach, although pea does not accumulate betaine; (b) the reaction rates on a Chl basis are 10-fold and 100-fold higher than, respectively, stromal BALDH activity and the in vivo rate of betaine synthesis in unstressed plants (9); (c) it is mediated by H₂O₂, an oxidant for which normal chloroplasts have efficient means of disposal (6); (d) betaine aldehyde is subject to ready chemical oxidation by a variety of conditions, including alkaline H₂O₂ (10). Taken with the observation that betaine synthesis in vivo does not require light (8), these considerations strongly imply that light-stimulated betaine aldehyde oxidation is an in vitro artifact. However, some in vivo flux via a light-dependent, H₂O₂-mediated oxidation cannot be ruled out, particularly in chloroplasts of highly stressed leaves in which the normal protective mechanisms against H₂O₂ may be disrupted (20).

We hypothesize that in both darkness and light the stromal BALDH is the primary means by which betaine aldehyde is converted in vivo to betaine, based on the high activity in the cellular compartment in which its substrate is produced, narrow substrate specificity, salt inducibility, and upon absence from a nonbetaine accumulating species. If this is true, classical or molecular-genetic approaches to inactivating the gene(s) for the stromal isozyme should lead to betaine-deficient plants suitable for genetic tests of the adaptive value of stress-induced betaine accumulation.

**LITERATURE CITED**

13. Liley RM, MP Fitzgerald, KG Rienhst, DA Walker 1975 Criteria of
BETAINE ALDEHYDE OXIDATION BY SPINACH CHLOROPLASTS

intactness and the photosynthetic activity of spinach chloroplast preparations. New Phytol 75: 1–10


17. OHITA-FUKUYAMA M, Y MIYAKE, S EMI, Y YAMANO 1980 Identification and properties of the prosthetic group of choline oxidase from Alcaligenes sp. J Biochem 88: 197–203


