ABSTRACT

The activity of cytidine 5’-diphosphate (CDP) choline: 1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) in developing soybean (Glycine max L. var Williams 82) seeds was 3 to 5 times higher in cotyledons grown at 20°C than in those grown at 35°C. Some characteristics of the enzyme from cotyledons cultured at 20 and 35°C were compared. In preparations from both growth temperatures, the enzyme showed a pH optimum of 7, K_m of 7.0 micromolar for CDP-choline, and an optimum assay temperature of 45°C. Both enzyme preparations were stimulated by increasing concentrations of Mg^{2+} or Mn^{2+}, up to 10 millimolar and 50 micromolar, respectively, though Mn^{2+} produced lower activities than Mg^{2+}. Enzymes from both 20 and 35°C show the same specificity for exogenous diacylglycerol. No metabolic effectors were detected by addition of heat treated extracts to the assay mixture. The above findings suggest that the higher enzymatic activity at 20°C can be attributed to a higher level of the enzyme rather than to the involvement of isozymes or metabolic effectors. Enzyme activity decreased rapidly during culture at 35°C, indicating a rapid turnover of the enzyme. The level of temperature modulation was found to be a function of seed developmental stage.

CDP-choline: 1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) is one of the key enzymes of lipid metabolism in plant tissues. It catalyzes the synthesis of PtdCho by trapping the acyl group in the 2-position of a diacylglycerol by the Kennedy pathway. A previous study from this laboratory found that cholinephosphotransferase and stearoyl-acyl carrier protein desaturase activities were 3 to 5 times higher in soybean cotyledons grown at 20°C than in those grown at 35°C (3); the in vitro activities of cholinephosphotransferase and stearoyl-acyl carrier protein desaturase were low compared to other enzymes in the pathway. The combination of temperature modulation and low activity were interpreted as indicating that these two enzymes were potential in vivo control sites for fatty acid desaturation (3). Similarly, in rye roots, it was shown that the incorporation of choline into PtdCho was higher in cold, 5°C-grown, than warm, 20°C-grown, roots (9). All three enzymes of the nucleotide pathway, including cholinephosphotransferase, showed higher activities in roots grown at low temperature. The frost tolerance of wheat can be increased by supplying choline chloride to the plant; this change is accompanied by an increase of PtdCho concentration in the membranes (6). In the latter examples, the role of increased PtdCho may be to enhance membrane fluidity at low temperatures (16).

In developing seeds, PtdCho also plays an important role in providing unsaturated fatty acids for triacylglycerol (17, 21). The formation of linoleic (18) and linolenic acids utilized for the synthesis of triacylglycerol is believed to take place on PtdCho through the desaturation of esterified oleoyl groups. It is, therefore, conceivable that temperature induced effects on PtdCho synthesis will affect the composition of soybean oil in the mature seed.

Cholinephosphotransferase from cotyledons cultured at 20 and 35°C was analyzed to determine the mechanism responsible for the temperature induced changes in activity observed in a previous study (3); results of these experiments are presented below.

MATERIALS AND METHODS

Chemicals

[Methyl-^{14}C]-cytidine diphosphocholine (CDP-choline, 52.1 mCi/mmol) was purchased from Du Pont-New England Nuclear (Boston, MA). 1,2-Diolein (18:1/18:1), 1,2-distearin (18:0/18:0) and 1,2-dipalmitin (16:0/16:0) were from Avanti Polar-Lipids (Pelham, AL). 1,2-dilinolein (18:2/18:2) and 1,2-dilinolenin (18:3/18:3) were obtained from Nu-Chek Preps. 1-Palmityl-2-oleoyl diacylglycerol (16:0/18:1), 1-oleoyl-2-palmityl diacylglycerol (18:1/16:0), 1-stearoyl-2-oleoyl diacylglycerol (18:0/18:1), and 1-stearoyl-2-linoleoyl diacylglycerol (18:0/18:2) were from Serva Research Laboratories (Port Huron, MI).

Preparation of the 104,000 g Fraction

The procedure to obtain the 104,000 g pellet was the same as previously reported (3). Briefly, developing seed pods of soybean (Glycine max L. var Williams 82) at the early stage of R5 (5.0-5.5 mm seeds) (5) were collected from plants grown in a greenhouse (31°C/26°C) and cultured in vitro for 20 h (unless otherwise specified) at 20 or 35°C (14). Seed homogenate was filtered through a 240 mesh nylon filter and 2

2 In the shorthand system used to identify fatty acids, the number preceding the colon indicates the number of carbon atoms and the number following the colon represents the number of double bonds. Pairs of fatty acids separated by a slash represent the components in the sn-1 and sn-2 positions, respectively, of a lipid molecular species.
centrifuged for 3 min at 8,000 g to remove cellular debris and the plastids. The supernatant was centrifuged for 45 min at 104,000 g to pellet the remaining organelles (11). The pellets were suspended in the homogenization medium at 1 mL/g fresh weight. The homogenization medium consisted of 150 mM Tricine [pH 7.5], 10 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 0.6 M sucrose and 1 mM dithiothreitol (11).

**Enzyme Assay**

The standard reaction mixture (12) for the assay of cholinephosphotransferase contained 50 mM Tris [pH 7], 10 mM MgCl₂, 1 mM dithiothreitol, 20 μM [¹⁴C]CDP-choline (13.25 mCi/mmol), and the 104,000 g fraction (at the final protein concentration of 20–80 μg/mL) in 500 μL reaction volume. The reaction was performed in a shaking water bath at 35°C for 20 min, and terminated by adding 50 μL of concentrated HCl. One mL of chloroform:methanol (2:1, v/v) was added, and the aqueous and organic phases were separated by centrifugation. The organic phase was washed once with 1 mL of methanol:water (1:1, v/v), dried, and the radioactivity quantitated by liquid scintillation counting. The formation of PtdCho was linear during the first 30 min of the reaction and proportional to the amount of protein in the 104,000 g fraction up to 100 μg/mL (data not shown). Protein was quantitated by micro-BCA (bicinchonic acid) assay (20). The enzyme activity was stable at least for 2 weeks when the preparation was stored at -80°C. Diacylglycerol, when added to the reaction mixture as a substrate, was dispersed in ice-cold 1% sodium taurocholate by ultrasonification for 15 min (15) with a model W-380 Sonicator (Heat Systems-Ultrasonics, Farmingdale, NY). The final concentrations of diacylglycerol and sodium taurocholate in the reaction mixture were 100 μM and 0.1%, respectively.

**RESULTS**

**Kinetic Properties of Cholinephosphotransferase**

Some kinetic characteristics of the enzyme were investigated using the 104,000 g fractions obtained from cotyledons grown at 20 or 35°C to determine if the difference in the enzyme activities at different temperatures was a result of isozymes with different properties. Cholinephosphotransferase from both preparations exhibited the same characteristics for pH optimum (Fig. 1), Mg²⁺ requirement (Fig. 2A), assay temperature effect (Fig. 3), and $K_m$ (CDP-choline) value (Fig. 4).

The enzyme activity showed a broad pH optimum between 6.5 and 8.0; with the highest activity at pH 7.0 (Fig. 1). The results are comparable to those reported in other plant tissues (4, 7, 12, 19). Assays using CHES buffer (pH 8.0–10.0) showed that the activity decreased sharply above pH 8.5 (data not shown). Cholinephosphotransferase requires Mg²⁺, or Mn²⁺, for activity (7). A concentration of 10 mM, or higher, Mg²⁺ was required for the full enzyme activity (Fig. 2A), while Mn²⁺ was effective at a much lower concentration (Fig. 2B). The enzyme activity increased up to 50 μM Mn²⁺, then decreased slightly at higher concentrations in 20°C cotyledons, but remained constant at higher concentrations in the 35°C preparation.
The specific activity of the enzyme from cotyledons was assayed at 20°C (data not shown). The addition of fully saturated species produced no change in activity, while addition of polyunsaturated species caused a slight decrease in enzyme activity in both 20°C- and 35°C-preparations.

These data indicate cholinephosphotransferase is kinetically identical in cotyledons grown at 20 and 35°C.

Role of Metabolic Effectors

The possibility that other cellular components might have caused the differences in the enzyme activity was tested. In this experiment, the 104,000 g supernatant or boiled 104,000 g pellets from 20°C-cotyledons was added to the reaction mixture for assays of the enzyme from 35°C-cotyledons, and vice versa. Supernatants were added to assays of the same homogenate (e.g., 20°C-104,000 g supernatant plus 20°C-104,000 g pellets) to examine the effect of cytosolic components on enzyme activity. The 35°C-enzyme was 97% as active in the presence of boiled 104,000 g pellets from 20°C-cotyledons as in their absence. The same was true in the reverse combination, 20°C-enzyme was 94% as activity in the presence of boiled 104,000 g pellets from 35°C-cotyledons as in their absence. The cytoplasmic fraction, however, inhibited the enzyme activity in both preparations to the same degree. Both 20°C- and 35°C-supernatants inhibited 20°C-enzyme prepa-

Molecular species specificity was analyzed by addition of 100 μM diacylglycerol, the other substrate of cholinephosphotransferase, to the assay mixture (Fig. 5). The specificity of 20°C- and 35°C-enzyme for different molecular species of diacylglycerol was revealed to be the same; in both preparations, 1,2-diolein (18:1/18:1) was the most effective molecular species tested. Enzyme activity was also increased by addition of molecular species containing oleic acid plus either palmitic acid or stearic acid. Although addition of 18:1/18:1 produced a greater increase in the activity of 35°C-enzyme, the difference in specific activity between 20°C- and 35°C-enzyme remained large. Diolein inhibited the enzyme activity at a concentration of 500 μM or above (data not shown). The addition of fully saturated species produced no change in activity, while addition of polyunsaturated species caused a slight decrease in enzyme activity in both 20°C- and 35°C-preparations.

Figure 5. Effect of different molecular species of exogenous diacylglycerol on cholinephosphotransferase activity. The enzyme was assayed in the standard reaction mixture plus either 100 μM diacylglycerol and 0.1% sodium taurocholate or 0.1% sodium taurocholate (control). Activity is expressed as per cent of the control treatment.

Figure 3. Effect of assay temperature on cholinephosphotransferase activity. Seed pods were cultured at 20°C or at 35°C, and the resulting enzyme extracts assayed in the standard reaction mixture were assayed at the indicated temperatures.

Figure 4. Lineweaver-Burk plots for the initial velocity of cholinephosphotransferase activity as a function of CDP-choline. The assay for both enzyme preparations was done at 35°C. Vₘₐₓ was 0.48 nmol/min/mg protein for 20°C-enzyme and 0.14 nmol/min/mg protein for 35°C-enzyme, while Kₘ was same (7.0 μM) for both preparations.
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Effects of Culture Time or Development Stage on Enzyme Activity

Figure 6 shows the change in activity of cholinephosphotransferase during the in vitro culture of soybean seed pods. The activity increased slightly up to 20 h and decreased thereafter in cotyledons grown at 20°C, while 35°C-grown seeds exhibited continuously decreasing enzyme activity. As discussed above, the decrease in 35°C-enzyme activity is not caused by induction of metabolic effectors; this indicates the decrease may be due to either increased turnover or decreased synthesis of the enzyme.

The magnitude of the decrease in cholinephosphotransferase activity from 35°C-cotyledons was found to be a function of the developmental stage of the seed (Fig. 7). When the seed pods of the later R5 stages were used for the experiment, the decrease of the enzyme activity during 20 h-culture was not so pronounced as when the younger seeds were used. The difference was even greater (about 10 times) in cultured cotyledons of R4 stage (data not shown).

DISCUSSION

Previous work indicates temperature may control the activity of enzymes in the nucleotide pathway for the synthesis of phosphatidylcholine (6, 9): the mechanism of this control is not known. Based on kinetic studies, Kinney et al. (9) suggested that the higher activity of cholinephosphotransferase, along with other enzymes in the pathway, in rye roots grown at 5°C versus 20°C might be due to an increase in enzyme concentration. The results presented above also suggest that the lower activity in 35°C-grown cotyledons may be due to a lower concentration of active enzyme in these tissues.

Growth temperature induced changes do not appear to involve induction of isoenzymes since the kinetic characteristics, like optimum pH (Fig. 1), cation requirement (Fig. 2), temperature coefficient (Fig. 3), $K_m$ for CDP-choline (Fig. 4), and the molecular specificity for diacylglycerol (Fig. 5) were essentially same in both preparations.

Addition of 104,000 g supernatants or boiled 104,000 g pellets from either 20°C or 35°C-cotyledons to the assay mixtures did not produce significant differences between 20 and 35°C enzyme. These data indicate it is unlikely that other cellular components may cause the observed difference in enzyme activity. Although there was actually an inhibitory factor in the 104,000 g supernatant from these tissues, the degree of inhibition was the same regardless of the growth conditions.

The apparent specificity of cholinephosphotransferase for diacylglycerol molecular species poses further questions on the in vivo function of this enzyme. The finding that cholinephosphotransferase is most active with substrates of 16:0/18:1 and 18:1/18:1 is consistent with the general concept that PtdCho is synthesized in a more saturated form, followed by sequential desaturation of its oleyl moieties. Similar results were obtained in spinach leaves (4), castor bean endosperms (13), and animal tissues (1). It was interesting to find that 18:1/16:0 diacylglycerol was also an effective substrate in soybeans, as well as in hamster hearts (1), because this molecular species is not normally available to the enzyme. Cholinephosphotransferase appears to be selective for diacylglycerol species with one or two double bonds in their fatty acyl moieties.

The rapid loss of cholinephosphotransferase activity in
35°C-cultures and the close relationship of modulation with the developmental stage of the seed (Figs. 6 and 7) are very intriguing; they suggest that growth temperature may control either the expression, turnover or inactivation of the enzyme in an extremely sensitive way.

LITERATURE CITED


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