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Expression and Characterization of Pea Chloroplastic Glyceraldehyde-3-Phosphate Dehydrogenase Composed of Only the B-Subunit[1]

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A cDNA fragment coding for the pea (*Pisum sativum* L.) chloroplastic glyceraldehyde-3-P dehydrogenase (EC 1.2.1.13) B-subunit and a truncated form corresponding in length to the A-subunit have been cloned into an expression vector, expressed in the absence of the A-subunit in a *gap*$^-$ *Escherichia coli* strain, purified, and studied. Like the isolated enzyme from higher plant chloroplasts, the recombinant enzymes have dual specificity for NADPH and NADH. The recombinant glyceraldehyde-3-P dehydrogenases have the same optimal pH as the enzyme isolated from pea chloroplasts. Like the native chloroplast enzyme, the recombinant B-subunit has a marked tendency to form large aggregates, whereas the truncated B-subunit exists as the tetramer. The recombinant B-subunit glyceraldehyde 3-P dehydrogenase is more sensitive to dithiothreitol than its truncated form. It seems likely that a different pair of cysteines is responsible for the redox sensitivity of the activity of the enzyme composed of B-subunits than the cysteine residues implicated in the modulation of the activity of the enzyme composed of A-subunits by previous work in this laboratory.

In chloroplasts G-3-P dehydrogenase (EC 1.2.1.13) catalyzes the reversible reduction and dephosphorylation of 1,3-bisphosphoglycerate to G-3-P using NADPH generated by PSI in the light. The enzyme is light- and DTT-activated (Buchanan, 1980; Anderson, 1986). It can use either NADP(H) or NAD(H) as the pyridine nucleotide substrate. In angiosperm chloroplasts there are two G-3-P dehydrogenase isozymes: one is composed of two A-subunits and two B-subunits (A_2B_2, isozyme I) and has a marked tendency to aggregate; the other is composed of four identical A-subunits (A_4, isozyme II) and does not aggregate (Cerff and Chambers, 1978, 1979; Ferri et al., 1978, 1990; Cerff, 1979; Pupillo and Faggiani, 1979; Scagliarini et al., 1993).

The A- and B-subunits are very similar, except that the B-subunit has a highly negatively charged C-terminal extension that contains two Cys groups and is not found in other G-3-P dehydrogenases (Shih et al., 1986, 1991; Brinkmann et al., 1989; Liaud et al., 1990; Baalmann et al., 1996) (Fig. 1). Both subunits are encoded in the nucleus (Cerff and Kloppstech, 1982; Shih et al., 1986). The existence of a B_4 tetramer in vivo has not been suggested. Baalmann et al. (1996) have developed an expression system for the A- and B-subunits of spinach (*Spinacia oleracea*) chloroplastic G-3-P dehydrogenase. Here we describe an expression system for the pea (*Pisum sativum*) chloroplastic G-3-P dehydrogenase B-subunit and for a truncated form corresponding in length to the A-subunit, and the characterization of the recombinant enzymes.

MATERIALS AND METHODS

Expression of Pea Chloroplast G-3-P Dehydrogenase in *Escherichia coli*

A cDNA coding for the pea (*Pisum sativum* L.) G-3-P dehydrogenase B-subunit (Brinkmann et al., 1989) (GenBank X15188) in plasmid pPsGapB was kindly provided by R. Cerff and M.F. Liaud. It was cloned into the *Xba*I/*Eco*RI sites of the expression vector pASK75 (Skerra, 1994) (Biometra, Tampa, FL) by PCR (Clackson et al., 1991) (Fig. 2). The sense primer 5′-AATATCATT**CTAGA**TAACGAGG GCAAAAAATGAAGTTGAAGGTAGCAATCAATG-3′ incorporated the *Xba*I site (in bold, which includes a stop codon, underlined, that terminates translation of the vector's β-galactosidase) followed by a ribosome-binding site and 22 bases of the N-terminal DNA sequence of the mature pea G-3-P dehydrogenase B-subunit. The antisense primer 5′-GCAACTGC**GAATTC**GGCTTGCTGGTAGAGG TAAT-3′ incorporated bases 29 to 48 after the stop codon of the gene and an *Eco*RI site (in bold).

To truncate the C-terminal extension of the B-subunit, we substituted the antisense primer 5′-GCAACTGC**GAA TTC**TTATGGCCATTTGTTTGCTACTA-3′, which added an *Eco*RI site (in bold) and a stop codon (underlined) after Pro-333. (Numbering according to *Bacillus stearothermophilus* G-3-P dehydrogenase, Brookhaven Protein Data Bank entry 1GD1; see Fig. 1.) Vent DNA polymerase (New England Biolabs) was used to ensure fidelity. *gap*$^-$ *E. coli* strain W3CG (Ganter and Plückthun, 1990), which cannot grow on unsupplemented LB media, was transformed

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Abbreviations: BΔ334, B-subunit truncated between Pro-333 and Gly-334; G-3-P, glyceraldehyde-3-P; LB, Luria-Bertani; PGA, 3-phosphoglyceric acid; P2GA, 1,3-bisphosphoglyceric acid.

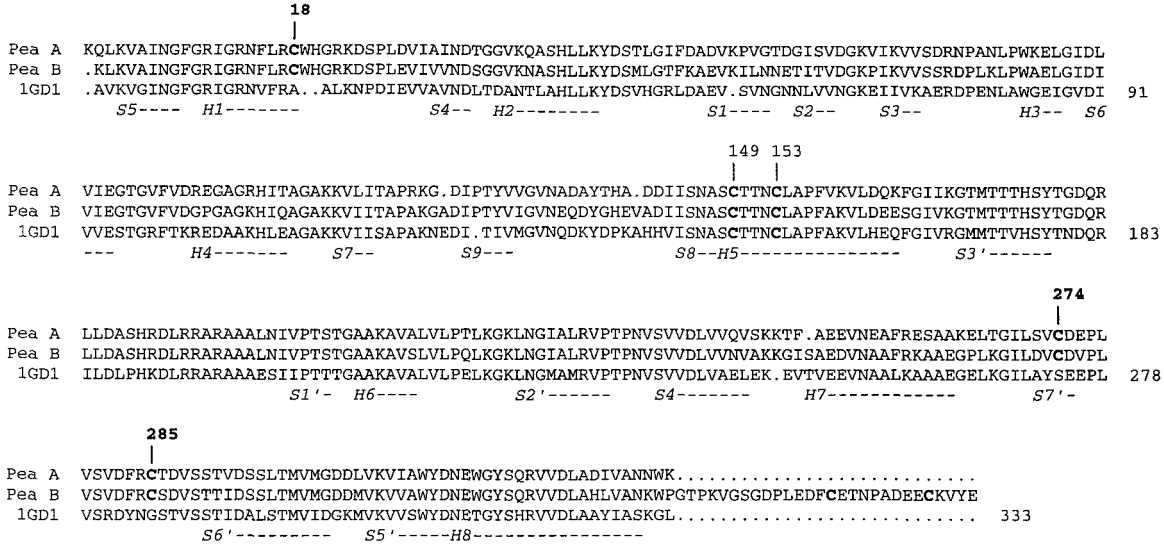

Figure 1. Sequence alignment of the pea A-subunit (Pea A; GenBank X15190) and B-subunit (Pea B; GenBank M55147) NADP-linked G-3-P dehydrogenases and the NAD-linked G-3-P dehydrogenase from *Bacillus stearothermophilus* (Biesecker et al., 1977) (Protein Data Bank 1GD1). Cys residues in the pea chloroplast enzyme are in boldface. The secondary structure from the Protein Data Bank file for the *B. stearothermophilus* enzyme is indicated with an *S* indicating a β-strand and *H* indicating an α-helix. Note that the numbering system for 1GD1 in the Brookhaven Protein Data Bank includes some additions and deletions. A multiple sequence alignment of the 105 G-3-P dehydrogenase sequences available in the GenBank, Swiss Protein, and Protein Data Bank data bases on January 1, 1996, was generated using the PILEUP program of the Wisconsin Sequence Analysis Package (version 8.0, Genetics Computer Group, Madison, WI) and optimized manually.

(Sambrook et al., 1989) with the reconstructed plasmids. About 3 to 5 μg of plasmid DNA was used for each 100 μL of competent cells.

After heat shock, 1 mL of M63 medium containing malate and glycerol (Ganter and Plückthun, 1990) was added for each 100 μL of cells, the cells were incubated at 37°C for 45 min, and 10 μg/mL tetracycline was added. After another 45 min at 37°C, the cells were concentrated by centrifugation and plated onto LB medium containing 50 μg/mL ampicillin and 10 μg/mL tetracycline. The plates were incubated at 37°C. Colonies appeared in 2 to 4 d. The DNA coding for the B- and the BΔ334-subunit in plasmids isolated from the bacteria after enzyme expression was sequenced by the staff at the DNA Sequencing Core Facility at the University of Illinois (Chicago). No changes in sequence were found.

Preparation of Crude Extract for Activity Assay

For preparation of crude extract on a small scale, liquid LB medium (3 mL) containing 50 μg/mL ampicillin and 10 μg/mL tetracycline was inoculated with single colonies from the selection plates. After 36 to 48 h of aerobic growth at 37°C, cells were collected by centrifugation (10,000*g*, 1 min in a microfuge), suspended in 0.5 mL of lysis buffer (50 mM Tris-HCl, pH 7.9, 1 mM potassium EDTA, 0.02% PMSF, and 0.1 mg/mL chicken lysozyme), and allowed to stand on ice for 1 h. Then, 7.5 μg of streptomycin in 150 μL of water was added to precipitate DNA. After 10 min (at 0°C) the mixture was centrifuged (10,000*g*, 5 min), and the supernatant was assayed for G-3-P dehydrogenase activity.

Purification of Recombinant Chloroplastic G-3-P Dehydrogenases

Two liters of LB medium containing 50 μg/mL ampicillin and 10 μg/mL tetracycline was inoculated with a 3-mL overnight culture of transformants. After overnight growth, the cells were harvested (3,000g, 10 min) and resuspended in 40 mL of 50 mM Tris-HCl, pH 7.9, 1 mM potassium EDTA, 0.02% PMSF, and 10 mM 2-mercaptoethanol. When the cells were fully resuspended, 10 mL of the resuspension buffer containing 5 mg of chicken lysozyme was added and the mixture was allowed to stand on ice for 2 h. Then, 1 g of streptomycin in 10 mL of water was added and the mixture was allowed to stand on ice for 30 min and then centrifuged (15,000g, 15 min). The supernatant was diluted 10 times with 0.01 M Tris-HCl, pH 7.9, containing 10 mM 2-mercaptoethanol and 1 mM potassium EDTA, and applied to a fast-flow DEAE cellulose column (25 mL) equilibrated with the same buffer.

After the column was washed with 50 mL of 0.01 M potassium phosphate, pH 7.0, 10 mM 2-mercaptoethanol, and 1 mM potassium EDTA, the enzyme was eluted in a linear 0.01 to 0.4 M potassium phosphate gradient (250 mL). The most active fractions were pooled, made 1 M in pH 7.0 potassium phosphate by the addition of 4 M potassium phosphate, pH 7.0, 10 mM 2-mercaptoethanol, and 1 mM potassium EDTA, and applied to a Phenyl Sepharose-4B column (5 mL, Serva) that had been equilibrated with 1 M potassium phosphate, pH 7.0, 10 mM 2-mercaptoethanol, and 1 mM potassium EDTA. This column was washed with 50 mL of the same buffer, and then developed with a 1 to

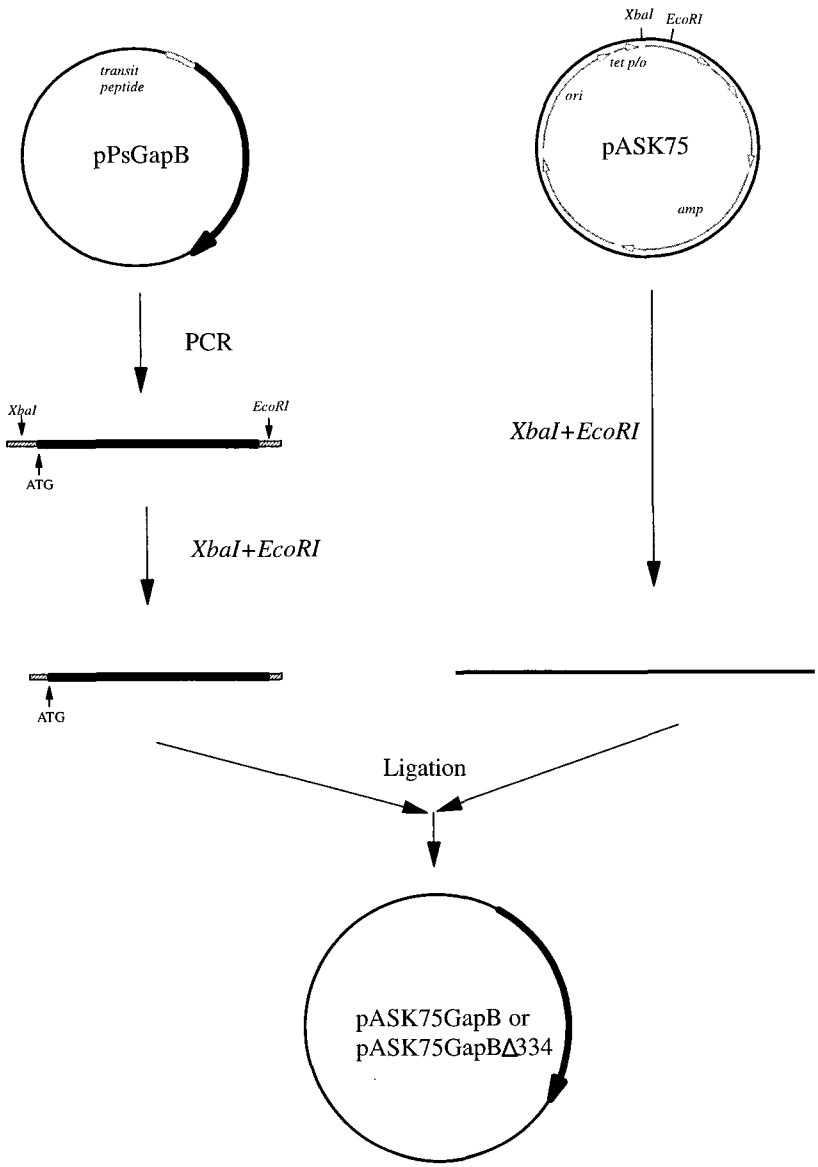

Figure 2. Subcloning of pea chloroplastic *gapB* into the expression vector pASK75. pPsGapB (Brinkmann et al., 1989) was from R. Cerff. *tet p/o*, Promoter/operator of the tetracycline-resistant gene; *amp*, ampicillin-resistant gene; *ori*, origin of replication.

0.01 M potassium phosphate gradient in 0.02% PMSF, 10 mM 2-mercaptoethanol, and 1 mM potassium EDTA (250 mL). The most active fractions were pooled. The enzymes were quite stable at 4°C. There was no loss of the C-terminal extension of the G-3-P dehydrogenase B-subunit even after 2 months. The final specific activities of the purified recombinant G-3-P dehydrogenases were 90 to 100 units/mg (Table I).

SDS-PAGE

SDS-PAGE was carried out according to the method of Weber and Osborn (1975) with the Phast-Gel system (Pharmacia). Protein was stained with Coomassie brilliant blue R.

Immunoblots

Crude extract from transformed cells was heated in a boiling water bath for 5 min in 2% (w/v) SDS, 50 mM Tris-HCl, pH 6.8, 1% (v/v) 2-mercaptoethanol, 10% glycerol, and 0.01% (w/v) bromphenol blue. Crude stromal extract, prepared as described in Anderson et al. (1995b), was treated in the same manner. Electrophoresis was carried out on 10 to 15% gradient polyacrylamide SDS-Phast gels using the Phast System (Pharmacia). After electrophoresis the protein on the gels was transferred thermally to Immobilon-P membranes (Millipore) and treated first with mouse anti-pea chloroplastic NADP-linked G-3-P dehydrogenase (Anderson et al., 1995a), and then with goat anti-mouse polyvalent immunoglobulins alkaline phosphatase conjugate (Sigma), and developed as in Anderson et al. (1995a).

Size-Exclusion Chromatography

The purified recombinant enzymes were subjected to gel filtration through a Superose-12 column (0.78 × 30 cm,

Table I. *Purification scheme for recombinant G-3-P dehydrogenase composed of the B- or the BΔ334-subunit*

Treatment	Total Activity[a]	Total Protein	Total Volume	Recovery	Specific Activity	Purification
	units	*mg*	*mL*	*%*	*units/mg*	*-fold*
Recombinant B-subunit						
Crude extract	1550	120	60	100	12.9	1
DEAE cellulose	871	20	28	56	44	3.4
Phenyl Sepharose-4B	727	7.2	40	47	101	7.8
Recombinant BΔ334-subunit						
Crude extract	2900	150	60	100	19.3	1
DEAE cellulose	1760	34	28	61	52	2.7
Phenyl Sepharose-4B	645	7	35	22	92	4.8

[a] Refers to NADP-linked activity.

Pharmacia) in a fast-protein liquid chromatography system. The column was equilibrated with 50 mM potassium phosphate, 10 mM 2-mercaptoethanol, and 1 mM potassium EDTA, pH 7.0. About 18 μg of protein in a volume of 300 μL was applied to the column. The flow rate was 0.25 mL/min and 0.375-mL fractions were collected.

G-3-P Dehydrogenase Activity Assay

The standard assay mixture for NADP-linked G-3-P dehydrogenase was 100 mM Tris-HCl, pH 7.8, 5 mM PGA, 2.5 mM ATP, 10 mM $MgCl_2$, 0.1 mM NADPH (NADH was substituted for NADPH in the assay for NAD-linked activity), and 4 units of yeast 3-phosphoglycerate kinase in a final 1-mL volume. The change in A_{340} was followed on a recording spectrophotometer (Cary 210 or 219, Varian, Sunnyvale, CA). The assay temperature was 23°C.

Protein Estimation

Protein was estimated by the method of Bradford (1976) with BSA as standard.

pH Optimum

Three different buffers were used in the determination of pH dependency: Mes (2-[*N*-morpholino]ethanesulfonic acid)-KOH, Hepes-KOH, and Ches (2-[*N*-cyclohexylamino]ethanesulfonic acid)-KOH. Buffer anions were 100 mM. The remaining components were as in the standard assay. pH was measured after activity assay (pH meter 28, Radiometer, Copenhagen).

Estimation of Kinetic Parameters

For estimation of the K_m for NAD(P)H, PGA was 5 mM and the NAD(P)H concentration was varied from 0.002 to 0.4 mM at 12 even-reciprocal intervals. All other components were as in the standard assay. In the reverse direction (oxidation of G-3-P) the assay mixture was 100 mM Hepes-KOH, pH 7.8, 1 mM neutralized sodium arsenate, G-3-P, and NADP or NAD. G-3-P was freshly neutralized and its concentration was determined enzymatically. When G-3-P was the varied substrate, NADP was 0.5 mM and NAD was 5 mM. The G-3-P concentration was varied from 0.01 to 5 mM at 12 even-reciprocal intervals. G-3-P was 5 mM when NAD(P) was the varied substrate. The NADP concentration was varied from 0.005 to 0.5 mM at 12 even-reciprocal intervals. NAD concentration was varied from 0.05 to 5 mM at 12 even-reciprocal intervals. The data were analyzed with the computer program of Hanson et al. (1967). Weighted mean values for K_ms and SEs were estimated using the reciprocal of the variance as the weighting factor.

Enzymes and Chemicals

Vent DNA polymerase was obtained from New England Biolabs, and restriction endonucleases and T4 DNA ligase from Life Technologies. The fast-flow DEAE cellulose and other biochemicals were obtained from Sigma. All other reagents were analytical reagent grade or the highest quality commercially available.

RESULTS AND DISCUSSION

Expression of Pea Chloroplastic G-3-P Dehydrogenase in *E. coli*

We constructed the plasmids pASK75GapB and pASK75GapBΔ334 by inserting the gene for GapB (Brinkmann et al., 1989) (GenBank X15188) and the truncated form into pASK75 (see "Materials and Methods"). After transformation of the *gap*$^-$ *E. coli* strain W3CG (Ganter and Plückthun, 1990) with the reconstructed plasmids, the bacteria were able to grow on LB medium. The enzymes are apparently expressed and functional in *E. coli*. The *Synechocystis* PCC 6803 NADP-linked G-3-P dehydrogenase has also been shown to function in *E. coli* (Valverde et al., 1997).

The recombinant enzymes in crude *E. coli* extracts cross-reacted with antiserum against chloroplastic G-3-P dehydrogenase after SDS-PAGE (Fig. 3). They moved as 42-kD (B-subunit) and 38-kD (BΔ334-subunit) protomers. There was another band at about 30 kD that cross-reacted with the antibody, which probably represented partially degraded enzyme.

Purification

The specific activities of the purified recombinant G-3-P dehydrogenase composed of the B-subunit or of the BΔ334-subunit were 90 to 100 units/mg (Table I), similar to that of G-3-P dehydrogenase isolated from pea chloroplasts

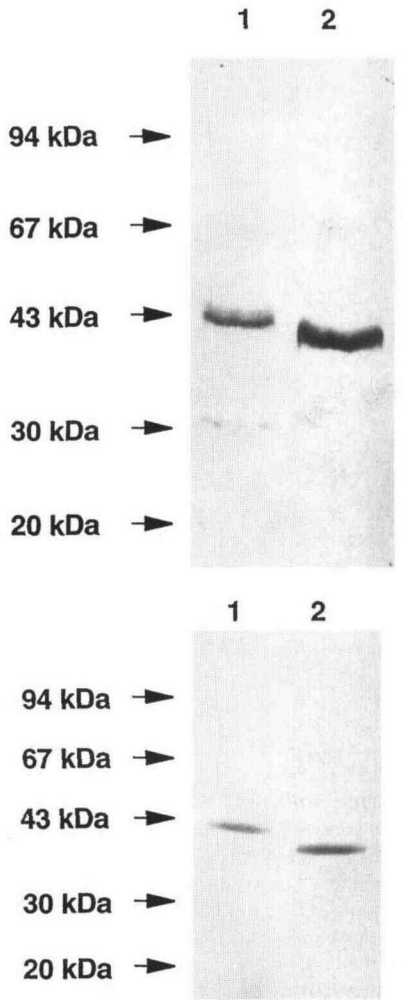

Figure 3. Top, Immunoblots of recombinant G-3-P dehydrogenases after SDS-PAGE. Lane 1, B-subunit in crude *E. coli* cell extracts; lane 2, BΔ334-subunit in crude *E. coli* cell extracts. Positions of molecular mass markers, phosphorylase *b* (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), and soybean trypsin inhibitor (20 kD), are indicated on the left. Bottom, SDS-PAGE of purified recombinant G-3-P dehydrogenase composed of the B-subunit and the BΔ334-subunit. PAGE was performed as above. Lane 1, B-subunit; lane 2, BΔ334-subunit. Molecular mass markers are as above.

(Anderson et al., 1995b). Yields were about 3.5 mg of purified enzyme per liter of culture. Similar yields were obtained by Baalmann et al. (1996) with the recombinant enzymes composed of the spinach chloroplastic A-subunit, the B-subunit, and the truncated form of the B-subunit, but the specific activity of their B-subunit enzyme was considerably lower (15–35 units/mg protein) than the specific activity of our recombinant G-3-P dehydrogenase B-subunit enzyme. The specific activity of their truncated enzyme was slightly higher (110–130 units/mg protein).

SDS-PAGE

After purification, the recombinant B-subunit enzyme and BΔ334-subunit enzyme were resolved into single bands on SDS-PAGE as shown in Figure 3. The estimated molecular mass of the recombinant B-subunit is 42 kD, which is the same as that of the B-subunit from pea chloroplasts (Cerff, 1979). The estimated molecular mass of the recombinant BΔ334-subunit is 38 kD, as expected for the truncated form.

Size-Exclusion Chromatography

When the recombinant G-3-P dehydrogenase B-subunit enzyme was subjected to gel filtration, activity corresponding to molecular sizes from 400 to 1000 kD was observed (Fig. 4). Similar results were obtained with the recombinant enzyme in crude extracts (data not shown). Like the A_2B_2 isozyme from chloroplasts (Cerff, 1978, 1979; Cerff and Chambers, 1978; Pupillo and Faggiani, 1979; Ferri et al., 1990), the recombinant enzyme composed only of B-subunit forms aggregates. The BΔ334-subunit activity peak emerged at the same position as the 146-kD rabbit muscle G-3-P dehydrogenase. Similar results were found with the recombinant spinach B-subunit enzyme and a truncated form of that enzyme (Baalmann et al., 1996). Apparently,

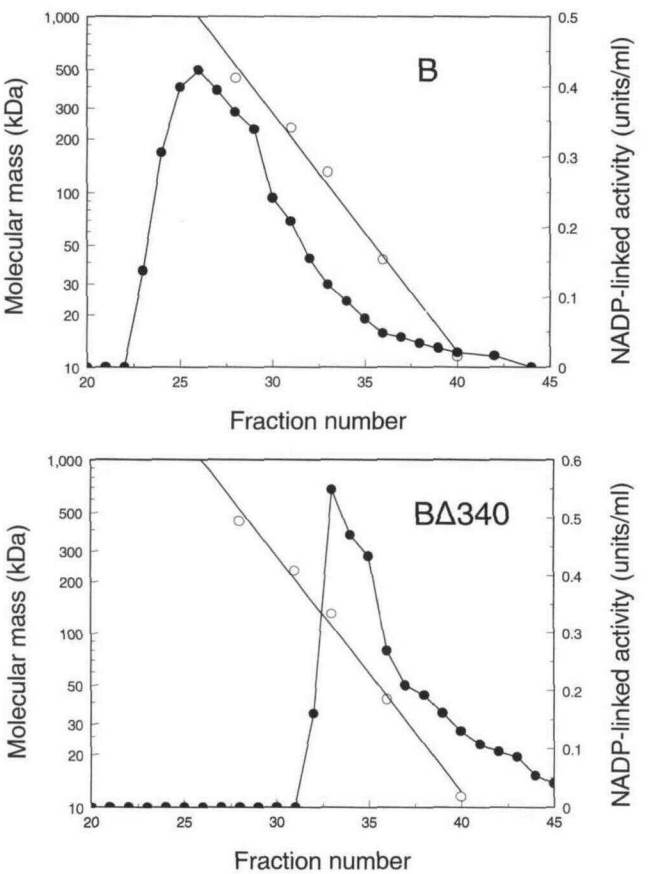

Figure 4. Size-exclusion chromatography of recombinant G-3-P dehydrogenases. Top, Profile for recombinant B-subunit enzyme. Bottom, Profile for recombinant BΔ334-subunit enzyme. The column was calibrated with respect to molecular size with the following proteins: horse spleen ferritin (445 kD), bovine liver catalase (230 kD), rabbit muscle G-3-P dehydrogenase (130 kD), *E. coli* phosphoglycerate kinase (41.4 kD), and horse heart Cyt *c* (11.7 kD).

the C-terminal extension is responsible for aggregation. In earlier experiments Zapponi et al. (1993) and Scheibe et al. (1996) found that proteolytic removal of the C-terminal extension from the spinach enzyme yielded a form of the enzyme that did not aggregate. They suggested that the C-terminal extension of the B-subunit is required for enzyme aggregation. Our results are consistent with theirs.

Dual NADP-/NAD-Linked Activity

We tested crude extracts from colonies of eight W3CG/pASK75GapB transformants and six W3CG/pASK75GapB-Δ334 transformants for G-3-P dehydrogenase activity (Fig. 5). All of the transformants had both NADP- and NAD-linked activity. Apparently, the enzyme coded by GenBank X15188 and its truncated form are able to utilize either pyridine nucleotide as the substrate. The average NADPH- to NADH-linked activity ratios were 4:1 for the B-subunit enzyme and 5.7:1 for the BΔ334-subunit enzyme. The activity ratio we found for the pea chloroplastic enzyme was 2.6:1 (Anderson et al., 1995b). Ratios of NADPH- to NADH-linked activity ranging from 0.1:1 to 3.5:1 have been reported for the purified chloroplastic enzymes from pea (Schulman and Gibbs, 1968; Melandri et al., 1970; McGowan and Gibbs, 1974; Pupillo and Faggiani, 1979), spinach (Yonuschot et al., 1970; Pupillo and Giulaini-Piccarri, 1975; Ferri et al., 1990; Trost et al., 1993), white mustard (Cerff, 1978), beet, buttercup, and *Arum italicum* (Pupillo and Faggiani, 1979). The somewhat higher NADPH to NADH activity ratio found for the recombinant B-subunit enzyme compared with the native chloroplastic enzyme suggests that the activity ratio of the B-subunit is higher than that of the A-subunit. It should be noted that the differences in the activity ratios reflect differences in K_m rather than k_{cat} (see below).

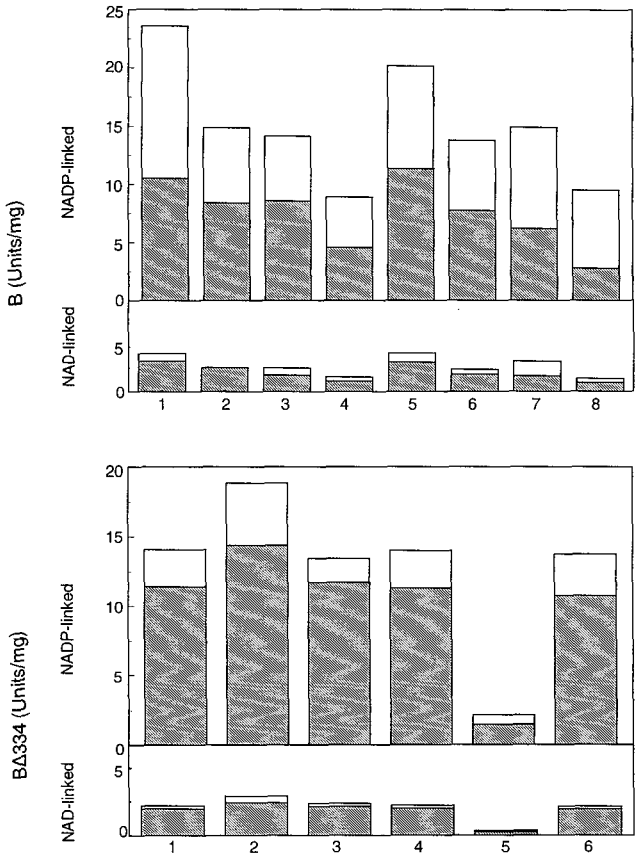

Figure 5. Effect of DTT on NADP- and NAD-linked activity of recombinant G-3-P dehydrogenases in crude *E. coli* extracts. Top, G-3-P dehydrogenase composed of B-subunits. Bottom, BΔ334-subunit. Activity before (gray) and after (white) treatment with 50 mM DTT for 30 min at 0°C is shown. Top frames, NADP-linked enzyme activity; bottom frames, NAD-linked enzyme activity. The average NADP-linked activity of recombinant B-subunit in the crude bacterial extracts was 8.7 units/mg; that of NAD-linked activity was 2.1 units/mg. The average NADP-linked activity of recombinant BΔ334 in crude extracts was 10.2 units/mg; that of NAD-linked activity was 1.8 units/mg.

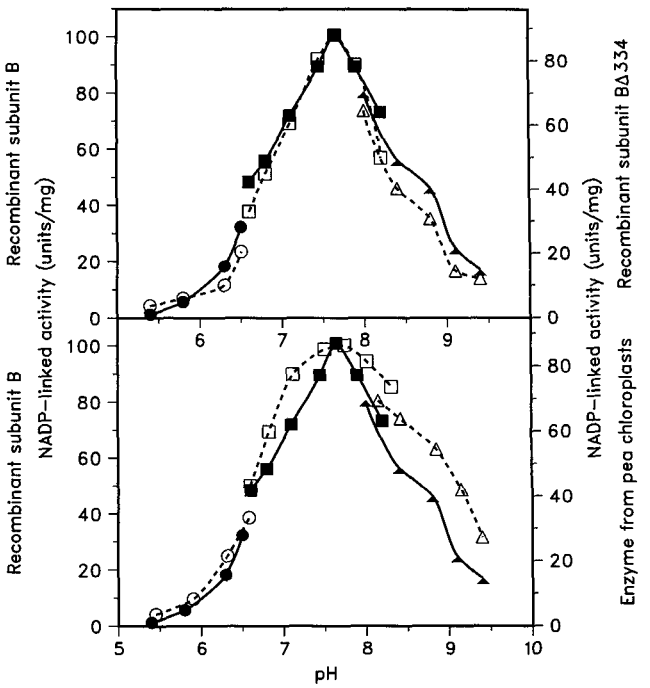

Figure 6. pH dependency of pea chloroplast G-3-P dehydrogenases. ○, Mes-KOH; □, Hepes-KOH; △, Ches-KOH. pH was measured after activity assay. Buffer anions were 100 mM. The remaining components were as in the standard assay. Top, pH dependency of recombinant G-3-P dehydrogenase composed of B-subunits (solid line and filled symbols) or BΔ334-subunits (dashed line and open symbols). Bottom, pH dependency of recombinant G-3-P dehydrogenase composed of B-subunits (solid line and filled symbols) and enzyme purified from pea chloroplasts (dashed line and open symbols) according to the method of Anderson et al. (1995b). Similar results were obtained in duplicate experiments.

pH Optima

Both of the recombinant G-3-P dehydrogenases and the enzyme isolated from pea chloroplasts are most active at about pH 7.65, but the enzyme from pea chloroplasts has a slightly broader pH-dependency curve (Fig. 6). It seems

possible that the A-subunit enzyme differs slightly from the B-subunit enzyme in its response to H^+ concentration. Clearly, the C-terminal extension does not affect the response of the B-subunit enzyme to pH. The enzyme from spinach chloroplasts has a similar pH optimum (see Ferri et al., 1978). The pH optima of two NADP-linked G-3-P dehydrogenases from the alga *Scenedesmus obliquus* are 7.5 (O'Brien and Powls, 1976). The pH optimum of the *Synechocystis* PCC 6803 enzyme is 8.2 (Valverde et al., 1997).

Kinetic Studies of Recombinant Chloroplastic G-3-P Dehydrogenases

Both the recombinant B-subunit and BΔ334-subunit G-3-P dehydrogenases have similar, significantly lower K_ms for NADP(H) than for NAD(H) (Table II). Cerff (1978) also reported that the K_m of G-3-P dehydrogenase from white mustard for NADPH is 13-fold lower than that for NADH. Ferri et al. (1978) reported that the K_m of the enzyme from spinach for NADP(H) is about 5-fold lower than that for NAD(H). There is no significant difference in the K_ms for NADP and NAD, and for NADPH and NADH, for the enzyme from *Synechocystis* PCC 6803 (Valverde et al., 1997).

In contrast, the K_m of the recombinant B-subunit enzyme for the reduced carbon substrate G-3-P was a little more than twice as high as the K_m of the truncated enzyme (Table II), suggesting that the C-terminal extension affects binding of the G-3-P. The K_m values reported previously for the pea chloroplastic enzyme were 0.115 mM (total G-3-P) with NADP as the pyridine nucleotide substrate and 0.117 mM with NAD as the substrate (Anderson et al., 1995b). Slightly higher values were reported by Schulman and Gibbs (1968). A lower apparent affinity for G-3-P would enhance CO_2 fixation. One of the effects of the C-terminal extension on the B-subunit may be a shifting of the steady-state equilibrium away from glycolysis and toward gluconeogenesis.

G-3-P dehydrogenase uses P2GA as an oxidized carbon substrate. In our assays this substrate was generated by yeast 3-phosphoglycerate kinase. If the kinase catalyzes rapid equilibrium between PGA and P2GA, it should be possible to estimate a K_m for P2GA for the dehydrogenase.

Table II. *K_ms of recombinant G-3-P dehydrogenase composed of the B- or the BΔ334-subunit*

Numbers in parentheses indicate number of determinations.

Substrate	K_m	
	B-Subunit	BΔ334-Subunit
	mM	
NADPH	0.038 ± 0.002 (2)	0.044 ± 0.002 (2)
NADP	0.077 ± 0.005 (2)	0.049 ± 0.004 (2)
G-3-P (total)[a]	0.52 ± 0.02 (3)	0.22 ± 0.02 (3)
G-3-P (free carbonyl)	0.018 ± 0.001 (3)	0.0076 ± 0.001 (3)
NADH	0.59 ± 0.05 (2)	0.64 ± 0.07 (2)
NAD	0.79 ± 0.05 (2)	0.80 ± 0.06 (2)
G-3-P (total)[b]	0.73 ± 0.05 (3)	0.35 ± 0.02 (3)
G-3-P (free carbonyl)	0.025 ± 0.002 (3)	0.012 ± 0.001 (3)

[a] With 0.5 mM NADP. [b] With 5 mM NAD.

Table III. *k_{cats} of recombinant G-3-P dehydrogenase composed of the B- or the BΔ334-subunit*

The forward direction is the direction of reduction of P2GA. Pyridine nucleotide concentrations were varied in k_{cat} estimations and PGA was 5 mM. The reverse direction is the direction of oxidation of G-3-P. Pyridine nucleotide concentrations were varied and G-3-P was 5 mM. Numbers in parentheses indicate number of determinations.

Reaction	k_{cat}	
	B-Subunit	BΔ334-Subunit
	s^{-1}	
NADP-linked		
Forward	89 ± 3 (2)	94 ± 3 (2)
Reverse	30 ± 1 (2)	29 ± 1 (2)
NAD-linked		
Forward	62 ± 4 (2)	66 ± 6 (2)
Reverse	31 ± 1 (2)	22 ± 1 (2)

In fact, K_ms for PGA are easily estimated using our assay mixture (which contains yeast phosphoglycerate kinase and recombinant enzyme), but when P2GA concentrations are calculated and used to estimate the K_m for P2GA, the error in the K_m values is very large (more than 30%). On Lineweaver-Burk plots there are two intersecting lines, one (high substrate concentrations) that gives a positive K_m value, and another (low substrate concentrations) that gives a negative K_m value (not shown). These mixed kinetics are probably indicative of substrate channeling between the yeast kinase and recombinant pea chloroplastic dehydrogenase (see Macioszek et al., 1990).

There is no significant difference between the k_{cats} of the NADP-linked activity of the recombinant B-subunit and the BΔ334-subunit enzymes, but for the NAD-linked reverse direction, the k_{cat} of the recombinant B-subunit enzyme is higher than that of the BΔ334-subunit enzyme (Table III). The ratios of the forward (gluconeogenic) and reverse (glycolytic) activities of the recombinant enzymes are about 3:1 when the pyridine nucleotide substrate is NADP(H). However, the ratio of the forward and reverse activity of the B-subunit enzyme is about 2:1 when the pyridine nucleotide substrate is NAD(H). For the BΔ334-subunit enzyme the ratio of the forward and reverse activity is about 3:1. In this case the extension would seem to favor glycolysis since the ratio of the forward to reverse reactions is lower for the NAD-linked activity of the recombinant B-subunit enzyme. This effect would not be expected to be metabolically significant. k_{cat} is an estimate of activity at infinite substrate concentrations, but substrate levels will be quite low in chloroplasts in the dark.

Effect of P2GA Generation Mixture

Baalmann et al. (1995) observed a 3-fold activation with P2GA over the activation of the spinach G-3-P dehydrogenase with DTT. We tested the effect of a P2GA-generating mixture on the recombinant enzymes in crude extracts (Table IV). The activity of the B-subunit enzyme was increased only by 18% over the DTT-activated activity. The activity of the BΔ334-subunit enzyme was not affected. (In every case P2GA was added along with the components of

Table IV. *The effect of P2GA generation mixture on NADP-linked recombinant G-3-P dehydrogenase activity in crude extracts*

The P2GA-generating mixture was according to Baalmann et al. (1995). In the treatment mixture, DTT was 50 mM, and the calculated P2GA concentration was 0.01 mM.

Treatment	G-3-P Dehydrogenase Activity	
	B-Subunit	BΔ334-Subunit
	units/mg	
DTT + P2GA	16	30
P2GA	9	28
DTT	14	28
Control	8	27

the P2GA-generating mixture.) Apparently, P2GA does not enhance the reductive activation of the recombinant B-subunit G-3-P-dehydrogenase.

Reductive Activation

We tested the effect of DTT on the activity of the recombinant enzymes in crude extracts (Fig. 5). Activation of NADP-linked activity in the eight recombinant B-subunit crude extracts ranged from 1.6- to 3.1-fold (the average activation was 2-fold). Activation of NADP-linked activity in the six recombinant BΔ334-subunit crude extracts ranged from 1.2- to 1.5-fold (the average activation was 1.3-fold) (Fig. 5). The NAD-linked activities were less sensitive to DTT than the NADP-linked activities. These experiments indicate that one or both of the Cys residues in the C-terminal extension to the B-subunit is involved in the reductive activation of the activity of the B-subunit. After purification the enzyme was no longer DTT activated. This might be the result of some conformational change during purification or of modification of a redox-regulatory Cys sulfhydryl (e.g. by oxidation to a sulfone).

Baalmann et al. (1996) found a 3- to 9-fold difference in the specific activities of the recombinant B-subunit enzyme from spinach and its truncated form. They suggested, on this basis and on the fact that the C-terminal extension appears to be necessary for aggregation, that reductive activation involves "C-terminal extension Cys-mediated aggregation." The higher specific activity of our recombinant B-subunit enzyme suggests that there is some other explanation for the difference in specific activities observed by Baalmann et al. (1996). We note that these workers did not confirm the sequence of the gene in the expression system.

We previously identified Cys-18 and Cys-285 as the residues most likely to form a redox-sensitive regulatory disulfide bond in chloroplast G-3-P dehydrogenase by comparative modeling (Li et al., 1994). We suggested that this interdomain disulfide would restrict the movement between the carbon substrate and the nucleotide-binding domains postulated to be required for catalysis (see Gerstein et al., 1994), with a resultant decrease in the activity of the enzyme. Reductive cleavage of the disulfide bond in the light would free the domains and result in an increase in activity. Consistent with our hypothesis, the *Chlamydomonas reinhardtii* chloroplast NADP-linked G-3-P dehydrogenase, which, aside from the active site Cys-149 and its near neighbor, Cys-153, contains only Cys-18 and Cys-285, is activated about 3-fold in the light in vivo and by DTT in vitro (Li et al., 1997). In addition, the enzymes from two red algae and two cyanobacteria, which have Cys-18 but lack Cys-285, are not redox sensitive (Pacold et al., 1995a, 1995b). Our present results, in contrast, offer no indication of a redox-sensitive regulatory disulfide in the BΔ334 mutant even though it contains both Cys-18 and Cys-285.

All attempts at expression of the A-subunit of the pea or Arabidopsis enzyme in the pASK75 system have failed. Therefore, we have not tested the redox sensitivity of the A_4 isozyme. Furthermore, although we were able to obtain expression of the B-subunit and its truncated form, we have not been able to obtain expression of mutants in which Cys-18, Cys-274, Cys-285, or the Cys residues in the extensions were changed to Ser. Therefore, we have not been able to compare the redox sensitivities of mutants lacking the implicated Cys residues and the recombinant B-subunit enzyme. Baalmann et al. (1996) were able to express the spinach A-subunit in a different expression system, but did not test the redox sensitivity of the enzyme.

Our current results indicate that the C-terminal extension is required for the redox-sensitivity of the enzyme composed of the B-subunit. They are not consistent with our modeling experiments or with our experiments with the *C. reinhardtii* enzyme in that Cys-18 and Cys-285 do not appear to form a redox-regulated disulfide bond in the B-subunit. It seems possible that Cys-18 and Cys-285 cannot form a disulfide bond in the B-subunit and that one of the disulfide-bond-forming Cys residues in the A-subunit has been replaced by one of the Cys residues in the C-terminal extension, or even that two new disulfides involving both of the Cys in the extension and Cys-18 and Cys-285 are present in the dark, inactive form of the enzyme.

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