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Functional studies of chloroplast glyceraldehyde-3-phosphate dehydrogenase subunits A and B expressed in *Escherichia coli*: formation of highly active A_4 and B_4 homotetramers and evidence that aggregation of the B_4 complex is mediated by the B subunit carboxy terminus

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Abstract

Chloroplast glyceraldehyde-3-phosphate dehydrogenase (phosphorylating, E.C. 1.2.1.13) (GAPDH) of higher plants exists as an A_2B_2 heterotetramer that catalyses the reductive step of the Calvin cycle. In dark chloroplasts the enzyme exhibits a molecular mass of 600 kDa, whereas in illuminated chloroplasts the molecular mass is altered in favor of the more active 150 kDa form. We have expressed in *Escherichia coli* proteins corresponding to the mature A and B subunits of spinach chloroplast GAPDH (GapA and GapB, respectively) in addition to a derivative of the B subunit lacking the GapB-specific C-terminal extension (CTE). One mg of each of the three proteins so expressed was purified to electrophoretic homogeneity with conventional methods. Spinach GapA purified from *E. coli* is shown to be a highly active homotetramer (50–70 U/mg) which does not associate under aggregating conditions *in vitro* to high-molecular-mass (HMM) forms of ca. 600 kDa. Since B_4 forms of the enzyme have not been described from any source, we were surprised to find that spinach GapB purified from *E. coli* was active (15–35 U/mg). Spinach GapB lacking the CTE purified from *E. coli* is more highly active (130 U/mg) than GapB with the CTE. Under aggregating conditions, GapB lacking the CTE is a tetramer that does not associate to HMM forms whereas GapB with the CTE occurs exclusively as an aggregated HMM form. The data indicate that intertetramer association of chloroplast GAPDH *in vitro* occurs through GapB-mediated protein-protein interaction.

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CTE, carboxy-terminal extension; HMM, high molecular mass; ATP, adenosine triphosphate; 3PGA, 3-phosphoglycerate; 1, 3bisPGA, 1,3-bisphosphoglycerate; HMM, high-molecular mass

Introduction

Eubacteria, archaebacteria, non-green algae and non-photosynthetic eukaryotes possess homotetrameric glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzymes [8, 13, 14]. By contrast, NADP-dependent GAPDH of the Calvin cycle in higher-plant chloroplasts (EC 1.2.1.13) occurs as an A_2B_2 heterotetramer. Chloroplast GAPDH has been extensively characterized with respect to structure and function for a number of plant sources [7, 9–12, 23, 30]. The A and B subunits of chloroplast GAPDH (GapA and GapB, respectively) are known have arisen via gene dupli-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number L76552 (GapA) and L76553 (GapB).

cation during chlorophyte evolution, their amino acid sequences share roughly 80% amino acid identity [6]. GapB differs from GapA (and all other GAPDH subunits) by the presence of a highly negatively charged C-terminal extension (CTE) of roughly 30 amino acids in length [6, 18], but the precise functional role of the B subunit has not been clarified.

The A_2B_2 enzyme from spinach chloroplasts has been studied in particular detail. It is activated by light in a redox-dependent manner [1, 12, 26] and the amino acid sequence of the purified enzyme is known [12]. Light activation of the spinach enzyme correlates to changes in aggregation state of the enzyme in isolated intact spinach chloroplasts. In the dark, the less active, ca. 600 kDa form of chloroplast GAPDH (probably a tetramer of tetramers) predominates that is partially converted in the light to the roughly four-fold more active, 150 kDa (tetrameric) form [1, 2, 26]. Conversion to the activated 150 kDa form *in vivo* is modulated under reducing conditions (electron pressure, thioredoxin) in the presence of ATP and 3PGA primarily by the resulting 1,3-bisphosphoglycerate (1, 3bisPGA) concentrations and is accompanied by a 20-fold increase of the affinity for the substrate 1, 3bisPGA [1, 2]. Aggregation of active chloroplast GAPDH to ca. 600 kDa forms can also be induced *in vitro* by addition of 140 μM NAD^+ [2, 24], and this property laid the foundation for the development of an efficient purification procedure [8].

It was recently shown that limited proteolysis *in vitro* with V_8 endoprotease removes C-terminal residues of both A and B subunits which might be involved in tetramer aggregation [31; R. Scheibe and E. Baalmann, unpublished]. In order to determine whether homomeric forms of chloroplast GAPDH are active and to clarify the function of the GapB-specific CTE, we have cloned and expressed mature spinach chloroplast subunits GapA, GapB, and GapB lacking the CTE under the control of bacteriophage T_7 promoter in *E. coli*. The plant enzymes so expressed were purified to homogeneity. Here we report the enzymatic activity of these homomeric proteins and their capacity to undergo NAD^+-induced intertetramer association.

Material and methods

Construction of plasmids

Isolation and manipulation of cDNA clones were performed by the methods described [21]. From a cDNA library of light-grown spinach [15], full-size clones for GapA and GapB were isolated by heterologous hybridization using the homologues from pea [6]. These were subcloned into pbluescript vectors (Stratagene) and sequenced by standard techniques [25].

PCR reactions were performed in 25 μl containing 0.5 U *Tli* polymerase (Serva) in the supplied buffer supplemented with 1 mM $MgCl_2$, 50 μM of each dNTP, 1.5 μM of each primer, and 1 ng of plasmid template. Thirty-five cycles of 1 min/93 °C, 1 min/50 °C and 2 min/72 °C were used. Primers used (5′-3′) were: A-For, TGCAC**CATATG**AAATTGAAGGTAGCAATCAATGGA; A-Rev, GCATA**GGATCC**TTACTGCCATTTGTTAGCAACAAT; B-For, TGCAC**CATATG**AAACTTAAGGTGGCAATCAATGG; B-RevS, GCAAT**GGATCC**TCACTTCCACTTGTTTGCTACCAAGTCTG; and B-RevL, GCAAT**GGATCC**TTTACTCGTAAAGTTTGCACTCC (restriction sites in bold face, underlined in Fig. 1). PCR products were purified by electroelution and DE-52 chromatography, digested with *Bam*HI and *Nde*I and ligated into *Bam*HI-*Nde*I cut pET-3a [28]. Transformants in *E. coli* nm522 which gave PCR products with the appropriate primers were sequenced to confirm reading frame fusions. Three plasmids so selected – $pGapA^M$, $pGapB^M$ and $pGapB^{M\Delta C}$ – encoding the mature subunits of GapA, GapB, and GapB lacking the carboxy-terminal extension, respectively, were transformed into *E. coli* BL21 [28] to yield strains $21GapA^M$, $21GapB^M$ and $21GapB^{M\Delta C}$.

Induction and lysis

200 ml of LB medium [25] containing 200 μg/ml ampicillin were inoculated with an over-night culture and allowed to grow at 37 °C. At an OD_{600} of 1.2, 50 ml of fresh medium were added and cells were induced with 0.4 mM isopropyl β-D-thiogalactoside. Two hours after induction, cells were harvested and resuspended in 100 ml of buffer A (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 14 mM 2-mercaptoethanol) with 140 μM NAD^+ and centrifuged. The pellet was resuspended in 25 ml buffer A with 140 μM NAD^+ and 100 μg/ml lysozyme. After incubation for 15 min at 30 °C, $MgCl_2$ was added to 10 mM and RNase and DNase were added to 1 μg/ml each. After shaking for 20 min, the suspension was centrifuged for 15 min at 4 °C and 10 000 × g.

GapA

```
tcaagactcaagactcaagactcaagactcaagagcATGGCTTCCAACATGCTCTCTATC   60
                                    M  A  S  N  M  L  S  I

GCTAACCCGTCTCTCCGGGTTTACAACAAGGGATTTTCTGAATTTTCTGGTCTACACACC  120
A  N  P  S  L  R  V  Y  N  K  G  F  S  E  F  S  G  L  H  T

TCTTCCCTTCCCTTTGGTCGCAAGGGCTCTGATGACTTGATGGCTTTTGTGTCCTTCCAA  180
S  S  L  P  F  G  R  K  G  S  D  D  L  M  A  F  V  S  F  Q
                                                   <||>
ACTAATGCTGTTGGAGGGAAGAGAAGTAGCCAGAATGGAGTAGTTGAGGCAAAATTGAAG  240
T  N  A  V  G  G  K  R  S  S  Q  N  G  V  V  E  A  K  L  K
                                                M
GTAGCAATCAATGGATTCGGAAGAATTGGAAGGAATTTCCTAAGGTGTTGGCATGGTAGG  300
V  A  I  N  G  F  G  R  I  G  R  N  F  L  R  C  W  H  G  R

AAGGACTCTCCCCTTGATGTTGTGGTGATCAACGACACTGGAGGTGTGAAACAAGCTTCC  360
K  D  S  P  L  D  V  V  V  I  N  D  T  G  G  V  K  Q  A  S

CACCTTCTAAAGTACGACTCCATCCTCGGAACATTTGATGCTGATGTTAAGACTGCTGGG  420
H  L  L  K  Y  D  S  I  L  G  T  F  D  A  D  V  K  T  A  G

GATAGTGCAATCTCTGTTGATGGCAAAGTCATCAAAGTTGTTTCTGACAGAAACCCTGTC  480
D  S  A  I  S  V  D  G  K  V  I  K  V  V  S  D  R  N  P  V

AACCTTCCTTGGGGGGACATGGGCATAGACTTGGTTATTGAAGGAACAGGTGTGTTCGTA  540
N  L  P  W  G  D  M  G  I  D  L  V  I  E  G  T  G  V  F  V

GACAGAGATGGTGCAGGTAAGCACTTACAAGCAGGGGCTAAGAAAGTGTTGATCACTGCC  600
D  R  D  G  A  G  K  H  L  Q  A  G  A  K  K  V  L  I  T  A

CCAGGAAAGGGTGACATACCAACCTATGTCGTTGGTGTCAACGAGGAAGGTTACACCCAT  660
P  G  K  G  D  I  P  T  Y  V  V  G  V  N  E  E  G  Y  T  H

GCTGACACCATCATCAGTAACGCTTCCTGTACCACTAACTGCTTGGCTCCCTTTGTTAAG  720
A  D  T  I  I  S  N  A  S  C  T  T  N  C  L  A  P  F  V  K

GTTCTTGACCAGAAATTTGGCATCATCAAGGGAACCATGACTACAACCCACTCCTACACG  780
V  L  D  Q  K  F  G  I  I  K  G  T  M  T  T  T  H  S  Y  T

GGAGACCAAAGACTCTTGGATGCAAGCCACAGGGATCTTAGACGTGCAAGAGCAGCATGT  840
G  D  Q  R  L  L  D  A  S  H  R  D  L  R  R  A  R  A  A  C

TTGAACATTGTCCCAACATCAACAGGTGCAGCTAAGGCTGTTGCATTAGTCCTTCCTAAC  900
L  N  I  V  P  T  S  T  G  A  A  K  A  V  A  L  V  L  P  N

CTAAAGGGAAAGCTTAACGGAATTGCTCTTCGTGTTCCAACACCAAATGTATCTGTGGTA  960
L  K  G  K  L  N  G  I  A  L  R  V  P  T  P  N  V  S  V  V

GACCTTGTCGTACAAGTCAGCAAGAAAACCTTCGCTGAAGAAGTTAATGCTGCCTTCAGA 1020
D  L  V  V  Q  V  S  K  K  T  F  A  E  E  V  N  A  A  F  R

CAGTCTGCAGACAATGAACTCAAAGGCATTCTCTCAGTCTGTGATGAGCCTCTTGTGTCC 1080
E  S  A  D  N  E  L  K  G  I  L  S  V  C  D  E  P  L  V  S

ATTGACTTCAGATGCACTGATGTTTCCTCAACTATTGATTCTTCATTGACCATGGTTATG 1140
I  D  F  R  C  T  D  V  S  S  T  I  D  S  S  L  T  M  V  M

GGAGATGATATGGTCAAAGTTATTGCATGGTATGATAATGAGTGGGGTTACTCTCAAAGA 1200
G  D  D  M  V  K  V  I  A  W  Y  D  N  E  W  G  Y  S  Q  R

GTTGTTGATCTTGCTGACATTGTTGCTAACAAATGGCAGTAAattcatctatcatgttaa 1260
V  V  D  L  A  D  I  V  A  N  K  W  Q  *

tcaaaaccaaactcactcttttctctcattcatcatacttagtacgtacaattatcacca 1320

attttcccatgttgctactacttaattgttttcatctttttgtaataagagataactttc 1380

caaatttgtatagagca                                            1397
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GapB

```
taaaagaaccaacaATGGCTTCTCATGCAGCTCTAGCTCCTTCTAGAATCCCTGCAAGTA   60
              M  A  S  H  A  A  L  A  P  S  R  I  P  A  S  T

CAAGGCTCGCGTCTAAGGCTTCTCAGCAGTACTCCTTTCTTACTCAATGCTCCTTTAAGA  120
 R  L  A  S  K  A  S  Q  Q  Y  S  F  L  T  Q  C  S  F  K  R

GACTCGACGTAGCTGACTTTTCTGGATTACGATCCAGCAACAGCGTGACATTCACAAGGG  180
 L  D  V  A  D  F  S  G  L  R  S  S  N  S  V  T  F  T  R  E

AGGCTTCATTTCATGATGTCATAGCTGCACAGCTAACCACCAAGCCTACAGGAGCAGCAC  240
 A  S  F  H  D  V  I  A  A  Q  L  T  T  K  P  T  G  A  A  P
                  <||>
CTGTTAGGGGTGAAACAGTGGCCAAACTTAAGGTGGCAATCAATGGATTTGGACGCATTG  300
 V  R  G  E  T  V  A  K  L  K  V  A  I  N  G  F  G  R  I  G
                   M
GTAGGAATTTCCTTAGATGCTGGCACGGCCGCAAAGACTCACCCCTTGATGTTGTTGTTG  360
 R  N  F  L  R  C  W  H  G  R  K  D  S  P  L  D  V  V  V  V

TCAACGACAGTGGAGGTGTCAAGAGTGCAACACACTTGCTCAAGTATGACTCCATACTAG  420
 N  D  S  G  G  V  K  S  A  T  H  L  L  K  Y  D  S  I  L  G

GAACCTTCAAAGCCGATGTGAAAATTATTGACAACGAGACCTTTTCCATCGATGGCAAGC  480
 T  F  K  A  D  V  K  I  I  D  N  E  T  F  S  I  D  G  K  P

CTATCAAGGTTGTCTCTAACAGGGACCCTCTTAAACTTCCTTGGGCTGAACTAGGCATTG  540
 I  K  V  V  S  N  R  D  P  L  K  L  P  W  A  E  L  G  I  D

ATATTGTTATTGAGGGAACAGGAGTCTTTGTTGATGGCCCTGGAGCTGGAAAACACATTC  600
 I  V  I  E  G  T  G  V  F  V  D  G  P  G  A  G  K  H  I  Q

AAGCTGGTGCCAAGAAAGTAATCATCACTGCTCCGGCAAAGGGTTCTGATATCCCGACCT  660
 A  G  A  K  K  V  I  I  T  A  P  A  K  G  S  D  I  P  T  Y

ATGTTGTTGGGGTAAACGAGAAAGACTATGGTCACGATGTTGCAAACATCATAAGCAATG  720
 V  V  G  V  N  E  K  D  Y  G  H  D  V  A  N  I  I  S  N  A

CTTCTTGCACCACCAACTGTCTGGCTCCATTTGTGAAGGTCTTGGATGAAGAATTAGGAA  780
 S  C  T  T  N  C  L  A  P  F  V  K  V  L  D  E  E  L  G  I

TTGTGAAGGGGACAATGACCACCACTCACTCCTACACTGGTGACCAGAGGCTGTTGGATG  840
 V  K  G  T  M  T  T  T  H  S  Y  T  G  D  Q  R  L  L  D  A

CTTCTCACAGGGACTTGAGAAGAGCCAGGGCTGCGGCATTGAACATAGTCCCAACCAGTA  900
 S  H  R  D  L  R  R  A  R  A  A  A  L  N  I  V  P  T  S  T

CTGGTGCAGCCAAGGCTGTATCTCTAGTCTTACCCCAACTTAAGGGAAAGCTCAATGGAA  960
 G  A  A  K  A  V  S  L  V  L  P  Q  L  K  G  K  L  N  G  I

TTGCCCTTCGTGTCCCAACACCTAACGTGTCTGTTGTTGATCTTGTTGTCAACATTGAGA 1020
 A  L  R  V  P  T  P  N  V  S  V  V  D  L  V  V  N  I  E  K

AGGTAGGTGTCACAGCTGAGGACGTAAACAATGCCTTCAGGAAAGCAGCAGCAGGTCCAT 1080
 V  G  V  T  A  E  D  V  N  N  A  F  R  K  A  A  A  G  P  L

TGAAGGGTGTGTTGGATGTGTGCGACATCCCCCTCGTGTCAGTTGATTTCAGGTGTTCTG 1140
 K  G  V  L  D  V  C  D  I  P  L  V  S  V  D  F  R  C  S  D

ATTTCTCATCTACAATCGATTCATCACTCACTATGGTAATGGGTGGTGATATGGTTAAGG 1200
 F  S  S  T  I  D  S  S  L  T  M  V  M  G  G  D  M  V  K  V

TCGTTGCTTGGTACGACAATGAGTGGGGTTACAGCCAACGGGTGGTGGACTTGGCAGACT 1260
 V  A  W  Y  D  N  E  W  G  Y  S  Q  R  V  V  D  L  A  D  L

TGGTAGCAAACAAGTGGCCGGGATTGGAGGGATCAGTTGCAAGCGGAGATCCATTGGAAG 1320
 V  A  N  K  W  P  G  L  E  G  S  V  A  S  G  D  P  L  E  D
                K  *     -                    -        -  -
ATTTCTGCAAGGACAACCCTGCTGATGAGGAGTGCAAACTTTACGAGTAAaaaaagatgt 1380
 F  C  K  D  N  P  A  D  E  E  C  K  L  Y  E  *
       +  -           -  -  -        +        -
gatcaactttacacttctttttggttattctgtatttggcctattacaaggctgtacatt 1440

tgattttatagcatcagcttctcattta                                 1669
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Figure 1. Sequences of spinach GapA and GapB and proteins expressed in *E. coli*. Primer target sites are underlined. Amino acid differences to *in vivo* mature subunits are indicated in bold. Noncoding regions are indicated in lower case. Transit peptide regions are indicated in italics, precursor processing sites as known from the sequence of the isolated proteins [12] are indicated with '<||>'. Stop codons are indicated with asterisks, positively and negatively charged residues in the CTE of GapB are indicated with '+' and '–', respectively.

Purification of expressed enzymes

All steps were performed at 4 °C. The cleared supernatant was applied at 1 ml/min to a red Sepharose column (Procion Red, Merck) equilibrated in buffer A. The column was washed at the same rate with 20 ml of buffer A containing 5 mM NAD^+, proteins were eluted at the same rate with 20 ml of buffer A containing 5 mM $NADP^+$. All fractions of the eluate with absorbance at 280 nm were pooled and concentrated to 5 ml with a 30K Microsep device (Filtron). The retentate was applied at 1 ml/min to a Source 15Q column (Merck) equilibrated with buffer B pH 8.5 (20 mM Tris-HCl, 14 mM 2-mercaptoethanol, 140 μM NAD^+) on an FPLC system (Pharmacia). Proteins were eluted at 1 ml/min in a 40 ml gradient of 0 to 500 mM NaCl and pH 8.5 to 8.0. Fractions of 0.4 ml were collected and assayed for NADPH-dependent activity.

Active fractions were pooled (and if neccessary concentrated to <5 ml with a 30K Microsep device) and subjected to FPLC gel chromatography on a 1.6 cm × 60 cm Superdex-200 column equilibrated with buffer C (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM KCl, 14 mM 2-mercaptoethanol, 140 μM NAD^+). Application and elution were performed at 1.5 ml/min. Void volume was 40 ml, 0.5 ml fractions were collected. The column had been calibrated using cytochrome *c* (12.5 kDa), chymotrypsinogen A (25 kDa), ovalbumin (45 kDa), bovine serum albumin (68 kDa), aldolase (158 kDa), catalase (240 kDa) and ferritin (450 kDa) as standards. Fractions containing NADPH-dependent GAPDH activity were pooled and concentrated with a 30K Microsep device. Glycerol was added to 50% v/v and samples were stored at –20 °C.

Native molecular mass determination and enzyme assay

200 μg purified enzyme was applied to an equilibrated Superdex-200 column as described above. After a void volume of 40 ml, fractions of 0.5 ml were collected, assayed for protein amount by the method of Bradford [4] (Serva) and for NADPH-dependent activity. Spinach chloroplast GAPDH preparations for comparison in SDS-PAGE were prepared as in [23]. Other molecular and biochemical methods were performed as described [25]. NADPH-dependent GAPDH activity was assayed by the method described in Baalmann *et al.* [2].

Results

From full-size cDNA clones isolated for GapA and GapB expressed in spinach leaves, we amplified regions encoding the respective mature chloroplast subunits and brought these under the control of the T_7 promoter for expression in *E. coli*. Figure 1 shows the sequence of spinach GapA and GapB cDNAs in addition to the proteins expressed in *E. coli* strains 21GapA^M, 21GapB^M and 21GapB$^{M\Delta C}$. The deduced amino acid sequences are identical to the sequences determined directly from the isolated chloroplast enzyme [12]. Strains 21GapA^M and 21GapB^M express proteins corresponding exactly to the mature A and B subunits of spinach chloroplast GAPDH, respectively, except that the N-terminal lysine residue is preceeded by a methionine in each case. Strain 21GapB$^{M\Delta C}$ expresses a derivative of spinach GapB^M, GapB$^{M\Delta C}$, which lacks the highly charged carboxy-terminal extension characteristic of all higher-plant GapB proteins [6, 18].

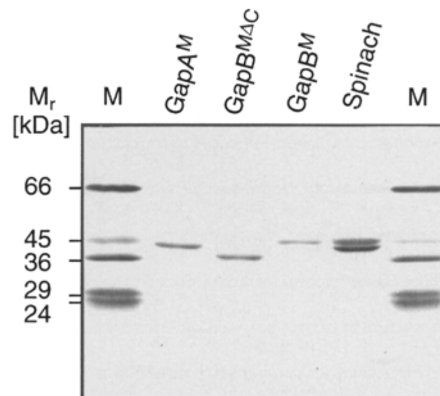

Figure 2. Coomassie-stained SDS-PAGE of $NADP^+$-dependent spinach chloroplast GAPDH subunits purified from expressing *E. coli* strains in comparison to the ioslated *in vivo* enzyme. M, molecular mass standards. Lanes GapA^M, GapB$^{M\Delta C}$, and GapB^M contain 1 μg each of the purified $NADP^+$-dependent GAPDH from the corresponding expressing *E. coli* strains. Two μg of purified chloroplast GAPDH prepared from isolated spinach chloroplasts as described in [1] were coelectrophoresed in the right lane for comparison. GapB$^{M\Delta C}$ migrates slightly faster than expected from the calculated molecular mass. Sizes of molecular mass standards (kDa) are indicated.

The spinach subunits so expressed were purified from *E. coli* crude extracts by affinity chromatography on red Sepharose, anion-exchange chromatography and gel filtration. The *E. coli* BL21 strain lacking expression plasmids possessed no detectable NADPH-dependent GAPDH activity. The final preparations of GapA^M, GapB^M and GapB$^{M\Delta C}$ are electrophoretically pure as shown in Figure 2. The relative molecular masses (M_r) observed in SDS-PAGE for GapA^M and GapB^M (Fig. 2, lanes 3 and 5) were indistinguishable from those for the A and B subunits of the holoenzyme purified from spinach chloroplasts (Fig. 2, lane 2). Although it has a M_r identical to that of GapA^M, GapB$^{M\Delta C}$ migrates in SDS-PAGE with a M_r of ca. 36 kDa (Fig. 2, lane 4), faster than expected on the basis of the calculated M_r of 39 kDa for the expressed protein. All three proteins were somewhat less stable in storage than the isolated enzyme from spinach, but showed no signs of either proteolysis or loss of activity during purification.

Typically, 0.4 – 1 mg of purified spinach subunits were obtained from 600 ml culture. Interestingly, preparations of each of the individual subunits were highly

enzymatically active with NADPH. $GapA^M$ had a specific activity of 50 to 70 U/mg. Preparations of $GapB^M$, which possesses the charged C-terminal extension, had 15 to 35 U/mg (comparable to the activity of the unactivated intact enzyme), whereas $GapB^{M\Delta C}$ preparations, which lack the C-terminal extension, contained the highest specific activities observed, 110 to 130 U/mg (i.e. comparable to the activated intact enzyme from spinach).

Purified enzyme preparations from the expressing strains were subjected to FPLC gel chromatography in buffer C containing 140 μM NAD^+ on a calibrated Superdex-200 column. $GapA^M$ eluted exclusively as a 150 kDa tetramer (Fig. 3), with no detectable 600 kDa form. The same is true for $GapB^{M\Delta C}$. By contrast, $GapB^M$ eluted exclusively as a high-molecular-mass form of roughly 470 kDa, with no detectable 150 kDa component.

To show that the aggregation of $GapB^M$ is NAD^+-dependent and reversible, 60 μg of 470 kDa $GapB^M$ were concentrated to 0.12 μg/ml, desalted on a NAP5 column (Pharmacia) with 1 ml of buffer D (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM KCl) to remove NAD^+ and applied to the Superdex-200 column equilibrated with buffer D. Protein and $NADP^+$-GAPDH activity eluted in buffer D exclusively as a 150 kDa form (data not shown). The calculated specific activity of the most concentrated fraction was in the range of 100–200 U/mg, but this value should be taken with caution because the protein concentration (ca. 4 μg/ml) in this fraction bordered on the detection level for the method.

Discussion

During the course of plant evolution, the gene for chloroplast GAPDH was transferred from plastids to the nucleus, where it came under the regulatory hierarchy of the nuclear transcription machinery, acquired a transit peptide for reimport of the mature subunit into the organelle of its genetic origin, and underwent duplication entailing acquisition of the CTE by the B subunit [17, 18]. Here the genes have been returned to a eubacterial genetic apparatus, the transit peptid has been removed and the CTE deleted.

Homomeric chloroplast GAPDH enzymes are active

Proteins expressed in *E. coli* corresponding to the mature subunits of spinach chloroplast GAPDH (GapA and GapB) in addition to a C-terminal deletion variant of GapB which lacks the CTE all possess NADPH-dependent GAPDH activity. The activity observed can be attributed exclusively to the expressed spinach proteins since (1) no NADPH-GAPDH activity was detected in controls lacking plasmids, (2) endogenous *E. coli* GAPDH is an NAD^+-dependent homotetramer of 36 kDa subunits and [5] which possesses no NADPH-dependent GAPDH activity [19]. Furthermore, since no contaminating *E. coli* GAPDH could be observed in final preparations of the expressed spinach chloroplast subunits (Fig. 3), we can conclude that the NADPH-dependent activity derives exclusively from homomeric forms of the spinach subunits, demonstrating conclusively that chloroplast GAPDH consisting solely of A or B subunits can freely associate (at least in *E. coli*) to active, functional enzymes.

Spinach $GapA^M$ expressed in *E. coli* and purified to homogeneity differs from the native chloroplast GapA subunit at the most by the presence of an additional methionine at its N-terminus. The N-terminal methionine is removed from many but not all proteins expressed in *E. coli* [20]. The specificity of the *E. coli* aminopeptidase for Met-Lys-, present in the proteins we expressed, is lower than for other substrates [3]. Thus the aminoterminal methionine may still be present in the expressed GAPDH proteins, although we did not determine this by sequencing. Pure $GapA^M$ associates to a homotetrameric holoenzyme (Fig. 3) and possesses a specific activity of 5O–70 U/mg, roughly two-fold lower than that of the activated, 150 kDa form of the native spinach A_2B_2 enzyme (120 U/mg) [8] . Somewhat surprisingly, $GapB^M$ associates to an enzymatically active form. The specific activity of the B^M enzyme purified from strain $21GaPB^M$ (15–35 U/mg) is roughly four-fold lower than that of the activated form of the enzyme in chloroplasts [1]. Gel filtration of active B^M forms reveals that this subunit readily associates to a high molecular mass multimeric form of the enzyme (Fig. 3). Since GAPDH enzymes require a minimum of four subunits for activity (i.e. monomeric, dimeric or trimeric species are completely inactive), activity of B^M indicates the presence of (aggregated) $B^M{}_4$ species. Although the M_r determined for B^M aggregates in gel filtration (ca. 470 kDa) is lower than that observed for the heterotetramer isolated from spinach chloroplasts (ca. 600 kDa) [1, 26], we cannot tell whether this is due to association of less than four homotetramers, or whether $(B^M{}_4)_4$ (i.e. ca. 600 kDa) aggregates exist

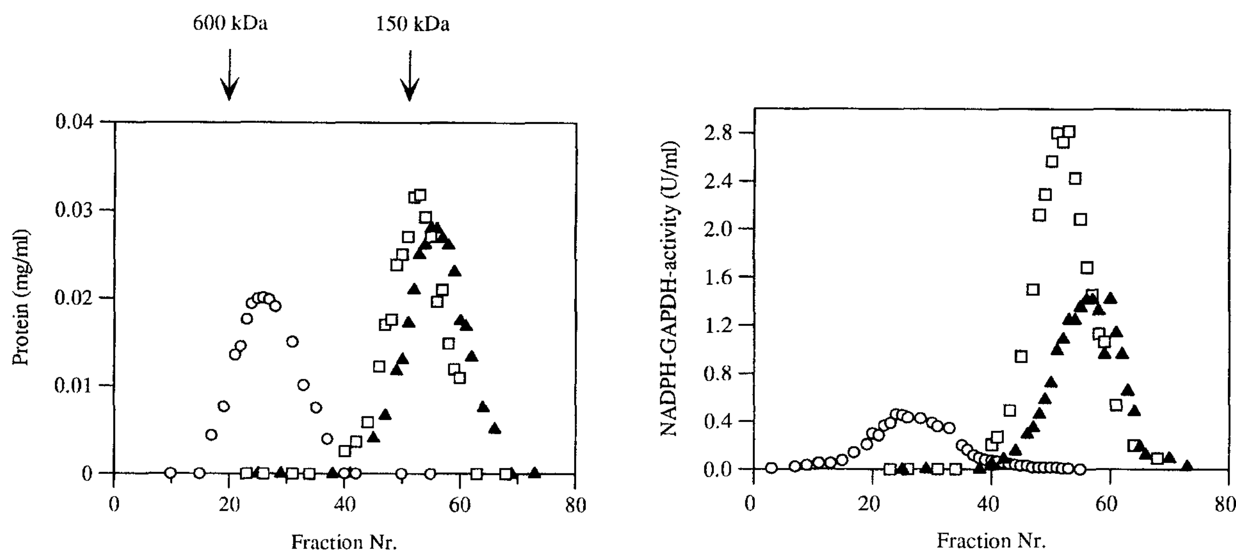

Figure 3. Superdex–200 elution profile of spinach chloroplast GAPDH expressed in *E. coli*. Conditions were as described in Materials and methods. Triangles, $GapA^M$; Squares, $GapB^{M\Delta C}$; Circles, $GapB^M$; A 200 μg portion of purified enzyme was applied in each case. **A** (left). Protein elution profile. **B** (right). Activity elution profile.

which however possess an anomalous chromatographic behaviour, mimicking a ca. 470 kDa form.

The CTE permits GapB aggregation to HMM forms

The three dimensional structure of GAPDH from numerous sources is known (see [29] and references therein), and in each case both N-terminus and C-terminus of each subunit are found exposed at the surface of the tetramer. It is thus very unlikely that the additional single N-terminal methionine possibly present in $GapA^M$, $GapB^M$ and $GapB^{M\Delta C}$ influences subunit interactions within tetramers. The conspicuous location of the highly charged CTE, at the surface of tetramers suggested to us, however, that it might be involved in tetramer interactions. Since $GapB^M$ readily aggregates to multimeric complexes, we asked: Does $GapB^M$ without the CTE also aggregate?

$GapB^{M\Delta C}$ differs from B^M in that the CTE of the spinach B subunit was removed and replaced by a single lysine residue at the position corresponding to the *in vivo* C-terminus of GapA (Fig. 1). Most surprisingly, $GapB^{M\Delta C}$ forms homotetramers (Fig. 3) which possess a specific activity equal to that of the heterotetramer isolated from spinach chloroplasts (110–130 U/mg). Yet, in contrast to $GapB^M$, $GapB^{M\Delta C}{}_4$ does not aggregate to forms of >150 kDa under aggregating conditions (Fig. 3). Since both $GapB^{M\Delta C}{}_4$ and $A^M{}_4$ are active but do not aggregate in the presence of NAD^+, whereas B^M does, these findings very strongly suggest that the ability of electrophoretically homogeneous preparations of spinach (and probably all higher-plant) chloroplast GAPDH to form NAD^+-induced aggregates of >150 kDa is mediated exclusively by the CTE of the B subunit. Interestingly, the roughly four-fold difference in specific activity observed between $GapB^{M\Delta C}{}_4$ and the aggregate (ca. 470 kDa) form of B^M is very similar to that observed between activated (150 kDa) and unactivated (600 kDa) forms of the enzyme isolated from spinach chloroplasts.

Higher-plant chloroplast GAPDH: A_2B_2 and A_4 or only A_2B_2?

The present findings indirectly address the still unresolved question of whether or not A_4 forms of the enzyme occur *in planta*. Whereas earlier work on purified chloroplast GAPDH from higher plants had indicated that two isoenzymes exist (isoenzyme I, an A_2B_2 heterotetramer, and isoenzyme II, an A_4 homotetramer) [8], the (still widely held) view that A_4 forms exist was questioned as evidence accumulated favoring the view that 'isoenzyme II' arises through partial proteolysis during preparation, and is merely an '$A_2B'_2$' heterotetramer in which the 29 amino acid carboxy-terminal extension (CTE) of both B subunits has been removed [6]. Indeed, partial proteolysis *in vitro* of the B subunit yields a product which is electrophoretically

indistinguishable from the A subunit [31; R. Scheibe and E. Baalmann, unpublished]. The CTE of the B subunit is shown here to confer upon GAPDH tetramers the ability to aggregate to higher molecular mass forms. Since 150 kDa forms of the enzyme are not found in dark spinach chloroplasts, rather only 600 kDa forms [1, 26], it would appear that the A_2B_2 species predominates in spinach. On the other hand, the present data indicate that highly active $A^M{}_4$ chloroplast GAPDH ('isoenzyme I') is readily formed in *E. coli*. $GapA_4$ tetramers might exist *in vivo*. But, since *in vitro* preparations of isoenzyme I may contain proteolytically produced $A_2B'_2$ forms which copurify and/or mimick A_4 species, clarification of the subunit composition of the enzyme *in vivo* will require antibodies which can discriminate between A and B subunits.

A regulatory function for the CTE

We have shown that NAD^+-dependent GAPDH aggregation is mediated by the CTE of the B subunit. If this NAD^+-dependent GAPDH aggregation mimics the aggregation seen *in vivo* which is thought to be light-regulated, then a very clear testable prediction would ensue. Namely, one would expect chloroplast GAPDH enzymes from plant sources which diverged from the higher-plant lineage prior to the gene duplication which gave rise to GapA and GapB of higher plants (1) (obviously) not to possess a GapB subunit and (2) (importantly) not to be regulated by light. The red algae *Chondrus crispus* and *Gracilaria verrucosa* possess chloroplast GAPDH enzymes which diverged from homologues for higher-plant GapA and GapB prior to the GapA/GapB split [16, 32]. The *Gracilaria* enzyme appears to consist of a single subunit (A_4), does not possess a CTE and, as was recently shown by Pacold *et al.* [22], is in fact not activated by light, and neither red algal enzyme is activated by DTT.

Although Pacold *et al.* [22] attributed those findings to the lack of specific 'conformation stiffening' (i.e. reducible and thus activating) cysteine residues in the GapA protein, their results would also be fully compatible with the view that the B-subunit mediates light activation via its CTE. The inactive, aggregated enzyme from dark spinach chloroplasts requires DTT or light-driven reduction prior to activation, implicating cysteine residues in this mechanism [1]. The CTE of known GapB sequences contains, in addition to other conserved sequence motifs, two conspicuous, strictly conserved cysteines (Fig. 4). It is possible that these cysteines in the CTE serve as the

```
Spinach      GapA  ...NKWQ*
Tobacco         "  ...NQWK*
Arabidopsis     "  ...NWK*
Pea             "  ...NWK*
Spinach      GapB  ...NKWPGLEGSVASGDPLEDFCKDNPADEECKLYE*
Tobacco         "  ...NKWPGSC·STGSGDPLDEFCKTNPADEECKVYE*
Arabidopsis     "  ...SKWPGAE·AVGSGDPLEDFCKTNPADEECKVYD*
Pea             "  ...NKWPGTP·KVGSGDPLEDFCETNPADEECKVYE*
                         •••      •••••  •   ••••••  •  •
                                  -  -- C    ---C+  -

                        <====C-Terminal Extension====>
```

Figure 4. Comparison of GapA and GapB C-termini and alignment of published carboxy-terminal extensions (CTEs). Sources of sequences are spinach (this paper), *Arabidopsis* [27], pea [6], and tobacco (published sequence corrected according to [6]. Stop codons are indicated by asterisks, '·' indicates a gap. Conserved residues are designated by '•', conserved charges by '–' and '+'. The two conserved cysteines in the CTE are given in bold face.

Chloroplast Subunit Expressed in *E. coli*		Subunit Association	Specific Activity [U/mg]
$GapA^M$	NAD(P)-Binding / Catalytic	Tetramer	50-70
$GapB^{M\Delta C}$	NAD(P)-Binding / Catalytic	Tetramer	110-130
$GapB^M$	NAD(P)-Binding / Catalytic / CTE (---Cys---Cys+-)	12-16mer	15-35

Figure 5. Summary of results for spinach chloroplast NADP-GAPDH subunits purified from expressing *E. coli* strains. Schematic representations of the functional domains in the protein and the conserved characteristics (charges, cysteine residues) of the CTE are indicated.

targets for redox-dependent priming of the enzyme for 1, 3bisPGA-mediated dissociation and activation. A schematic comparison of structure and specific activities of chloroplast GAPDH subunits expressed in *E. coli* here is summarized in Fig. 5. If the CTE-cysteine residues are in fact instrumental in light activation of the enzyme *in vivo*, we would predict them to be found in the GapB subunits of all light-activated chloroplast GAPDH enzymes.

Although in both regulation models (subunit-cysteine vs. CTE-cysteine-mediated aggregation) electron pressure (redox state) is involved in chloroplast GAPDH activation, our model would posit the necessity of the B-subunit and its CTE for light- dependent activation. To our knowledge, a CTE-mediated, associative mechanism of light-dependent activity modulation suggested here for chloroplast GAPDH has not been described for any other enzyme. It may represent a unique regulatory process of higher-plant chloroplasts.

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References

1. Baalmann E, Backhausen JE, Kitzmann C, Scheibe R: Regulation of NADP-dependent glyceraldehyde-3-phosphate dehydrogenase activity in spinach chloroplasts. Bot Acta 107: 313–320 (1994).
2. Baalmann E, Backhausen JE, Rak C, Vetter S, Scheibe R: Reductive modification and nonreductive activation of purified spinach chloroplast glyceraldehyde-3-phosphate dehydrogenase. Arch Biochem Biophys 324: 201–208 (1995).
3. Ben-Bassat A, Bauer K, Chang S-Y, Myambo K, Boosman A, Chang S: Processing of the initiation methionine from proteins: Properties of the *Escherichia coli* methionine aminopeptidase and its gene structure. J Bact 169: 751–757 (1987).
4. Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72: 248–254 (1976).
5. Branlant G, Branlant C: Nucleotide sequence of the *Escherichia coli gap* gene. Eur J Biochem 150: 61–66 (1985).
6. Brinkmann H, Cerff R, Salomon M, Soll J: Cloning and sequence analysis of cDNAs encoding the cytosolic precursors of subunits GapA and GapB of chloroplast glyceraldehyde-3-phosphate dehydrogenase from pea and spinach. Plant Mol Biol 13: 81–94 (1989).
7. Cerff R: Glyceraldehyde-3-phosphate dehydrogenase (NADP) from *Sinapis alba*: NAD(P) induced conformation changes of the enzyme. Eur J Biochem 82: 45–53 (1978).
8. Cerff R: Separation and purification of NAD- and NADP-linked glyceraldehyde-3-phosphate dehydrogenases from higher plants. In: Edelmann M, Hallick RB, Chua N-H (eds) Methods in Chloroplast Molecular Biology, pp. 683–694. Elsevier Biomedical Press, Amsterdam (1982).
9. Cerff R, Chambers S: Subunit structure of higher plant glyceraldehyde-3-phosphate dehydrogenases (EC 1.2.1.12 and 1.2.1.13). J Biol Chem 254: 6094–6098 (1979).
10. Ferri G, Comerio G, Iadarola P, Zapponi MC, Speranza ML: Subunit structure and activity of glyceraldehyde-3- phosphate dehydrogenase from spinach chloroplasts. Biochim Biophys Acta 552: 19–31 (1978).
11. Ferri G, Stoppini M, Iadarola P, Zapponi MC, Galliano M, Minchiotti L: Structural characterization of the subunits of spinach chloroplast glyceraldehyde-3-phosphate dehydrogenase (NADP). Biochim Biophys Acta 915: 149–156 (1987).
12. Ferri G, Stoppini MC, Meloni ML, Zapponi MC, Iadarola M: Chloroplast glyceraldehyde-3-phosphate dehydrogenase (NADP): Amino acid sequence of the subunits from isoenzyme I and structural relationship with isoenzyme II. Biochim Biophys Acta 1041: 36–42 (1990).
13. Fothergill-Gilmore LA, Michels PAM: Evolution of glycolysis. Progr Biophys Mol Biol 59: 105–238 (1993).
14. Hensel R, Zwickl P, Fabry S, Lang J, Palm P: Sequence comparison of glyceraldehyde-3-phosphate dehydrogenase from the three urkingdoms: evolutionary implication. Can J Microbiol 35: 81–85 (1989).
15. Henze K, Schnarrenberger C, Kellermann J, Martin W: Chloroplast and cytosolic triosephosphate isomerase from spinach: Purification, microsequencing and cDNA sequence of the chloroplast enzyme. Plant Mol Biol 26: 1961–1973 (1994).
16. Liaud M-F, Valentin C, Brandt U, Bouget FY, Kloareg B. Cerff R: The GAPDH gene system of the red alga *Chondrus crispus*: promoter structure, intron/exon organization, genomic complexity and differential expression of genes. Plant Mol Biol 23: 981–994 (1993).
17. Martin W, Brinkmann H, Savona C, Cerff R: Evidence for a chimaeric nature of nuclear genomes: eubacterial origin of eukaryotic glyceraldehyde-3-phosphate dehydrogenase genes. Proc Natl Acad Sci USA 90: 8692–8696 (1993).
18. Meyer-Gauen G, Schnarrenberger C, Cerff R, Martin W: Molecular characterization of a novel, nuclear-encoded, NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase in plastids of the gymnosperm *Pinus sylvestris* L. Plant Mol Biol 26: 1155–1166 (1994).
19. Michels S, Scagliarini S, Della Seta F, Carles C, Riva M, Trost P, Branlant G: Arguments against a close relationship between non-phosphorylating and phosphorylating glyceraldehyde-3-phosphate dehydrogenases. FEBS Lett 339:97–100 (1994).
20. Moguilevsky N, Varsalona F, Guillaume J-P, Gilles P, Bollen A, Robool K: Production of authentic proapolipoprotein A-I in *Escherichia coli*: strategies for the removal of the amino-terminal methionine. J Biotechnol 27: 159–172 (1993).
21. Nowitzki U, Westhoff P, Henze K, Schnarrenberger C, Martin W: Cloning of the amphibolic Calvin cycle/OPPP enzyme D-ribulose-5-phosphate 3-epimerase (E.C. 5.1.3.1) from spinach chloroplasts: Functional and evolutionary aspects. Plant Mol Biol 29: 1279–1291 (1995).
22. Pacold ME, Anderson LE, Li D, Stevens FJ: Redox sensitivity and light modulation of enzyme activity in the rhodophytes *Gracilaria tikvahiae* and *Chondrus crispus*. J Phycol 31: 297–301 (1995).
23. Pupillo P and Faggiani R: Subunit structure of three glyceraldehyde-3-phosphate dehydrogenases of some flowering plants. Arch Biochem Biophys 194: 581–592 (1979).
24. Pupillo P und Giuliani-Piccari G: The reversible depolymerization of spinach chloroplast glyceraldehyde-3-phosphate dehydrogenase. Interactions with nucleotides and dithiotreitol. Eur J Biochem 51: 475–482 (1975).
25. Sambrook J, Fritsch E, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
26. Scagliarini S, Trost P, Pupillo P, Valenti V: Light activation and molecular mass changes of NAD(P)-glyceraldehyde 3-phosphate dehydrogenase of spinach and maize leaves. Planta 190: 313–319 (1993).
27. Shih MC, Heinrich P, Goodman HM: Cloning and chromosomal mapping of nuclear genes encoding chloroplast and cytosolic glyceraldehyde-3-phosphate dehydrogenase from *Arabidopsis thaliana*. Gene 119: 317–319 (1992).
28. Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW: Use of T_7 RNA polymerase to direct expression of cloned genes. Meth Enzymol 185: 60–89 (1990).
29. Vellieux FMD, Hajdu J, Verlinde CLMJ, Groendijk H, Read RJ, Greenhough TJ, Campbell JW, Kalk KH, Littlechild JA, Watson HC, Hol WGJ: Structure of glycosomal glyceraldehyde-3- phosphate dehydrogenase from *Trypano-*

soma brucei determined from laue data. Proc Natl Acad Sci USA 90: 2355–2359 (1993).

30. Zapponi MC, Berni R, Iadarola P, Ferri G: Spinach chloroplast glyceraldehyde-3-phosphate dehydrogenase (NADP). Formation of complexes with coenzymes and substrates. Biochim Biophys Acta 744: 260–264 (1983).
31. Zapponi MC, Iadarola P, Stopponi M, Ferri G: Limited proteolysis of chloroplast glyceraldehyde-3-phosphate dehydrogenase (NADP) from *Spinacia oleracea.* Biol Chem Hoppe-Seyler 374: 395–402 (1993).
32. Zhou YH, Ragan MA: Cloning and characterisation of the nuclear gene encoding plastid glyceraldehyde-3-phosphate dehydrogenase from the marine red alga *Gracilaria verrucosa.* Curr Genet 26: 79–86 (1994).