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The C-terminal Extension of Glyceraldehyde-3-phosphate Dehydrogenase Subunit B Acts as an Autoinhibitory Domain Regulated by Thioredoxins and Nicotinamide Adenine Dinucleotide*

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The regulatory isoform of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a light-activated enzyme constituted by subunits GapA and GapB. The NADPH-dependent activity of regulatory GAPDH from spinach chloroplasts was affected by the redox potential ($E_{m,7.9}$, −353 ± 11 mV) through the action of thioredoxin *f*. The redox dependence of recombinant GapB ($E_{m,7.9}$, −347 ± 9 mV) was similar to native GAPDH, whereas GapA was essentially redox-insensitive. GapB mutants having one or two C-terminal cysteines mutated into serines (C358S, C349S, C349S/C358S) were less redox-sensitive than GapB. Different mutants with other cysteines substituted by serines (C18S, C274S, C285S) still showed strong redox regulation. Fully active GapB was a tetramer of B-subunits, and, when incubated with NAD, it associated to a high molecular weight oligomer showing low NADPH-dependent activity. The C-terminal GapB mutants (C358S, C349S, C349S/C358S) were active tetramers unable to aggregate to higher oligomers in the presence of NAD, whereas other mutants (C18S, C274S, C285S) again behaved like GapB.

We conclude that a regulatory disulfide, between Cys-349 and Cys-358 of the C-terminal extension of GapB, does form in the presence of oxidized thioredoxin. This covalent modification is required for the NAD-dependent association into higher oligomers and inhibition of the NADPH-activity. By leading to GAPDH autoinhibition, thioredoxin and NAD may thus concur to the dark inactivation of the enzyme *in vivo*.

Photosynthetic glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] catalyzes the reversible reduction of 1,3-bisphosphoglycerate (BPGA) to glyceraldehyde-3-phosphate in the presence of NAD(P)H. Two isoforms of GAPDH are found in the chloroplast stroma of higher plants: the A_4 isoform constituted by four identical GapA subunits, and the A_xB_x isoform formed by GapA and GapB subunits in apparent stoichiometric ratios (1, 2). The AB-isoform is finely regulated (3–8), whereas the A_4-isoform is known as the non-regulatory GAPDH (9). GapA is 336 amino acids long in *Spinacia oleracea* and 80% identical to the N-terminal moiety of GapB, which contains an additional C-terminal extension (CTE) of 28 residues (10, 11). The CTE includes two invariant cysteines and nine negatively charged amino acids, and is highly conserved among different plant species. Despite the high similarity between GapA and GapB, only the latter subunit seems to be involved in the regulatory mechanism of the enzyme (12, 13).

The activity of chloroplast GAPDH *in vivo* is stimulated by light (14). In the dark, regulatory GAPDH is found as a high molecular weight form endowed with low NADPH-dependent activity, although the activity in the presence of NADH is normal. Upon illumination, regulatory GAPDH is activated concomitant with dissociation of the high oligomers into lower molecular weight forms, mostly active tetramers (15, 16). Purified regulatory GAPDH shows a similar behavior: effectors such as NADP(H), BPGA, P_i, and ATP activate the enzyme by stabilizing the tetrameric conformation (A_2B_2), whereas NAD(H) does the opposite by inducing the enzyme to aggregate into a high oligomer (apparently a hexadecamer, A_8B_8) (3, 7, 8). The inter-exchange between A_2B_2 and A_8B_8 conformations has been suggested to be part of the light-activation mechanism (15, 16), but the *in vivo* aggregation of GAPDH into high molecular weight forms may involve still other proteins, such as the small regulatory protein CP12 and phosphoribulokinase (17–20). In principle, the two mechanisms are not reciprocally exclusive and might both contribute to the complex regulation of GAPDH and the Calvin cycle.

The NADPH-dependent activity of regulatory GAPDH is stimulated by reduced thioredoxin *f* (Trx *f*) (21, 22). In pea leaf GAPDH, the two conserved cysteines of the CTE can form a disulfide bridge (23) and are envisioned as likely targets for Trx regulation (12, 25). Although direct evidence of a regulatory role of this disulfide is lacking, it is well known that native or recombinant GapB truncated of the whole CTE ($GapB\Delta_{CTE}$) are fully active and unaffected by reductants and effectors (12, 13, 24), indicating that the fine-tuning of GAPDH regulation depends on the CTE. Accordingly, a model has been proposed in which the "light-activation" of photosynthetic GAPDH was first mediated by Trx through reduction of a disulfide bridge in the CTE and then by BPGA whose increased concentration in the light was thought to stabilize the active A_2B_2 conformation (8, 12).

In the present study we have investigated the redox regulation of photosynthetic GAPDH by a site-specific mutagenesis

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[1] The abbreviations used are: GAPDH, photosynthetic glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13); BPGA, 1,3-bisphosphoglycerate; CTE, C-terminal extension of subunit B of photosynthetic GAPDH; DTT, dithiothreitol; GapA, recombinant GAPDH, subunit A; GapB, recombinant GAPDH, subunit B; $GapB\Delta_{CTE}$, recombinant CTE-deleted GapB; IPTG, isopropyl-1-thio-β-D-galactopyranoside; Trx *f*, spinach thioredoxin *f*, recombinant form.

approach. Spinach GapA and GapB have been expressed in *Escherichia coli*, and all cysteines that could be potential targets for Trx action have been individually mutated to serines. Purified mutant proteins have been tested for their responsiveness to Trx *f* and to the substrate effectors, NADP and NAD. It is shown that Cys-349 and Cys-358, belonging to the CTE, are both required for Trx-dependent regulation of the GAPDH. The formation of the regulatory disulfide between these cysteines inhibits the enzyme and is essential for the NAD-dependent aggregation into high molecular weight forms. The results are discussed in light of the recent crystal structure of the A_4 isoform of spinach GAPDH complexed with NADP (26) and provide new insights into the molecular basis of GAPDH inactivation in the dark.

EXPERIMENTAL PROCEDURES

Production of Constructs for Recombinant GAPDH Subunits Expression—The cDNAs for *S. oleracea* GapA, GapB, and GapBΔ_{CTE}, inserted in the expression vector pET-14 and kindly provided by N. Wedell, were excised from the plasmid with endonucleases *Bam*HI and *Nde*I (Promega) and purified from low melting point agarose gel (0.8%, w/v) with the QIAquick gel extraction kit (Qiagen). Fragments were ligated into *Bam*HI- and *Nde*I-predigested pET-28a(+) (Novagen), and the ligation mixtures were used to transform *E. coli* cells, strain BL21(DE3).

Point mutations were introduced by PCR on the construct containing the coding region for GapB. Primers were as follows: C18SUp: 5′-TTG GTA GGA ATT TCC TTA GAA GCT GGC ACG GCG G-3′ and C18SDown: 5′-TCT TTG CGG CCG TGC CAG CTT CTA AGG AAA TCC-3′; C274SUp: 5′-TGT GTT GGA TGT GTC CGA CAT CCC CCT CG-3′ and C274SDown: 5′-GAG GGG GAT GTC GGA CAC ATC CAA CAC AC-3′; C285SUp: 5′-TGT CAG TTG ATT TCA GGA GTT CTG ATT TCT CAT CTA CAA TCG-3′ and C285SDown: 5′-ATT GTA GAT GAG AAA TCA GAA CTC CTG AAA TCA ACT GAC ACG-3′; C349SUp: 5′-CAT TGG AAG ATT TCT CCA AGG ACA ACC CTG-3′ and C349SDown: 5′-CAG GGT TGT CCT TGG AGA AAT CTT CCA ATG-3′; C358SUp: 5′-CAA CCC TGC TGA TGA AGA ATC CAA ACT TTA CGA G-3′ and C358SDown: 5′-CTC GTA AAG TTT GGA TTC TTC ATC AGC AGG GTT G-3′. A double mutant (C349S/C358S) was constructed by PCR using C358S as the template with primers C349SUp and C349SDown. PCR and transformation reactions were performed according to the QuikChange™ site-directed mutagenesis kit (Stratagene). Single colonies from each mutagenesis reaction were picked up, transferred into liquid culture (LB medium supplied with kanamycin, 50 μg/ml), and grown overnight at 37 °C under vigorous shaking (150–170 rpm). Plasmidic DNA have been extracted and sequenced before transferring the constructs into *E. coli* BL21(DE3).

Purification of Heterologously Expressed Recombinant Proteins and Native Regulatory GAPDH from Spinach Leaves—In a typical preparation, 20 ml of an overnight culture were transferred to 250 ml of fresh LB medium supplied with kanamycin (50 μg/ml). When the optical density of the culture at 600 nm reached 0.7 OD, expression was induced by addition of 0.4 mM IPTG. After 3 h of growth, cells were collected by centrifugation, washed with 25 mM potassium phosphate, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol (buffer A), and pelleted again before storage at −70 °C. Frozen cells were suspended in 25 ml of buffer A supplemented with 0.5 mM $NADP^+$ and lysated at 30 °C for 15 min in the presence of lysozyme (100 μg/ml). Nucleic acids were digested by incubation in 10 mM $MgCl_2$, 1 μg/ml RNase A, 1 μg/ml DNase I for 20 min at room temperature under continuous shaking. Cell debris was removed by centrifugation (15,000 × *g* for 15 min). An equal volume of cold acetone (−18 °C) was slowly added to the supernatant, and immediately centrifuged at −20 °C for 20 min at 20,000 × *g*. The corresponding pellet was resuspended in 25 ml of buffer A plus 0.5 mM $NADP^+$ and clarified by centrifugation (20 min, 20,000 × *g*). The supernatant was loaded onto a Q-Sepharose HP 16/10 anion-exchange column (Amersham Biosciences) previously equilibrated with buffer A. The column was thoroughly washed with buffer A before applying a linear potassium phosphate gradient corresponding to five column volumes (25–500 mM potassium phosphate for GapA and GapBΔ_{CTE}; 25–700 mM potassium phosphate for GapB and site-specific mutants of GapB). Active fractions of 1 ml were pooled and concentrated by ultrafiltration (Amicon cell, YM10 membrane). To remove the N-terminal His tag, recombinant proteins were incubated overnight at 20 °C in the presence of 10% glycerol (v/v) and thrombin protease (Amersham Biosciences). Following His tag cleavage, enzymes were diluted 10-fold in buffer A and loaded onto a second anion exchange column (Mono Q HR 5/5, Amersham Biosciences) equilibrated with buffer A. Linear salt gradients were 25–500 mM potassium phosphate in 15 ml for GapA and GapBΔ_{CTE}, and 25–700 mM potassium phosphate for GapB and relative mutants. Active fractions were collected, stored at +4 °C, and used within 2 days. Final preparations were electrophoretically pure as judged by SDS-PAGE performed on precast 12.5% Excelgel SDS Homogeneous (Amersham Biosciences).

Native regulatory GAPDH from spinach chloroplasts was purified according to ref. 9, except for the final size-exclusion chromatographic steps, which were omitted. Native GAPDH preparations were more than 90% pure as judged by SDS-PAGE. Purified GAPDH was stored at 4 °C in buffer A for no more than 2 days before use.

Assay of Enzyme Activity—GAPDH activity was monitored spectrophotometrically at 340 nm and 25 °C, in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 3 mM 3-phosphoglycerate, 1 mM EDTA, 5 units ml^{-1} of 3-phosphoglycerate kinase (from rabbit muscle, Sigma), 2 mM ATP, 0.2 mM NADPH or NADH. For calculations, an ϵ_{340} for NAD(P)H of 6.23 mM^{-1} was used. In redox titration experiments, the reaction mixture was the same except that Tris-HCl (50 mM, pH 7.5) was substituted by 20 mM Tricine-NaOH, pH 7.9.

Redox Titrations—Before each redox titration experiment, the enzyme preparation buffer was exchanged into 100 mM Tricine-NaOH, pH 7.9, by a passage through a Sephadex G-25 HiTrap Fast Desalting column (Amersham Biosciences). When necessary, enzyme solutions were either diluted or concentrated by ultrafiltration (Centricon, YM10 membrane) to give a final NADPH-dependent activity of 1500 nmol min^{-1} ml^{-1} (corresponding to an approximate enzyme concentration of 15 μg ml^{-1}).

Enzyme samples (40 μl) were incubated for 3 h at 25 °C with 2 μg ml^{-1} of recombinant spinach Trx *f*, kindly provided by P. Schürmann, and 20 mM DTT in various dithiol/disulfide ratios, in a total volume of 100 μl (27, 28). Following incubation, the NADPH-dependent activity (or in some cases the NADH-dependent activity) was assayed.

The titration results were fit by non-linear regression (CoStat, CoHort Software) to the Nernst equation setting the value of *n* at 2 (the disulfide/dithiol is expected to be a two-electron transfer process), according to Refs. 27 and 28. In some experiments, titration data were poorly fitted to the Nernst equation containing one redox component, but good fits were obtained with the Nernst equation containing two redox components. In these cases, the two $E_{m,7.9}$ values and the contribution of the two components have been optimized by the software.

Relative activities are expressed as a fraction of the activity measured after 3-h incubation with 20 mM reduced DTT and Trx *f* (2 μg ml^{-1}). Because the activity of any oxidized isoform never reached zero, we introduced into the Nernst equation a parameter representing the fraction of the relative activity that was subjected to redox regulation (*i.e.* the difference between the relative activity of the fully reduced form and the relative activity of the fully oxidized form). In each regression analysis, the exact value of this parameter (between 0 and 1) was optimized by the software.

The calculations of the $E_{m,7.9}$ values of the regulatory disulfides were based on a value of $E_{m,7.9}$ for the couple reduced/oxidized DTT of −380 mV (29, 30). Based on this value, the calculated E_h of the different incubation mixtures containing varying ratios of reduced and oxidized DTT ranged between −402 and −283 mV. Each titration experiment has been repeated on two independent enzyme preparations at least. The $E_{m,7.9}$ values are given as means ± S.D. (when omitted for the sake of clarity, S.D. values were between 5 and 15 mV).

Size-exclusion Chromatography—The aggregation state of purified recombinant proteins in the presence of either NADP or NAD was analyzed by size-exclusion chromatography on a Superdex 200 HR 10/30 column connected to a Smart System (Amersham Biosciences). The column was calibrated with standard globular proteins (Gel Filtration LMW and HMW Calibration kit, Amersham Biosciences). A linear relationship between log(apparent molecular mass) and elution volume was obtained in the range between 13.7 (ribonuclease A) and 669 kDa (thyroglobulin). The column was equilibrated with 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 14 mM β-mercaptoethanol, 150 mM KCl plus 0.2 mM NAD (associating conditions) or 0.2 mM NADP (dissociating conditions). Before loading, purified proteins were desalted in 100 mM Tricine-NaOH, pH 7.9, and incubated with 0.2 mM NAD or 0.2 mM NADP for 2 h on ice. Chromatographic runs were performed at a constant flow rate of 0.5 ml min^{-1}. Absorbance was monitored on-line at 280 and 260 nm. Fractions of 0.5 ml were collected, and the activity was determined by the standard assay with NADPH. Precise elution volumes were recorded in correspondence of the 280-nm absorbance peak, and apparent

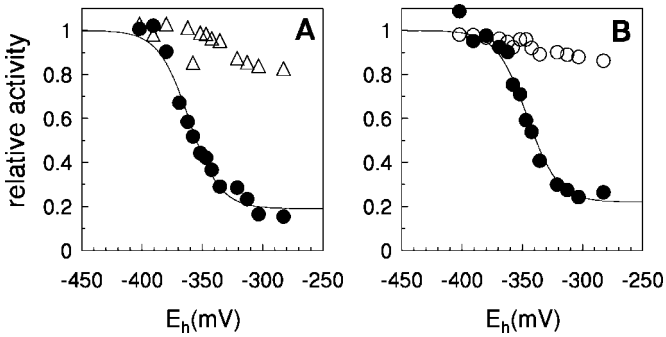

FIG. 1. **Redox titration of the activity of photosynthetic GAPDH following incubation with Trx *f* and oxidized/reduced DTT.** *A*, native regulatory GAPDH purified from spinach chloroplasts, NADPH-dependent activity (●), and NADH-dependent activity (△). *B*, NADPH-dependent activity of recombinant GapB (●) and GapA (○).

molecular masses were calculated on the basis of the column calibration.

RESULTS

Heterologous Expression and Purification of Spinach GAPDH Subunits—For the present study, spinach GAPDH subunits A and B (GapA, GapB) have been individually expressed in *Escherichia coli* cells and purified to homogeneity before characterization. The IPTG-induced expression of both GapA and GapB was strong, resulting in prominent bands in denaturating gel electrophoresis of whole extracts of transformed *E. coli* cells (not shown). Although heterologously expressed GapA and GapB included an N-terminal His tag provided by the expression vector (pET28, Novagen), any attempt to use metal affinity chromatography for protein purification was unsuccessful because of the strong inactivating effect of the eluant (imidazole) on the enzyme. We therefore developed a different purification method consisting of (i) acetone fractionation of the cell lysate followed by (ii) a first anion exchange column (Q-Sepharose), (iii) the His tag removal by thrombin treatment, and (iv) a second anion exchange on Mono Q. Final preparations obtained by this procedure were electrophoretically pure and enzymatically active (80–130 μmol min^{-1} mg^{-1}). Average yields varied between 0.9 (GapA) and 1.3 (GapB) mg of pure protein per liter of liquid culture.

Thioredoxin-mediated Redox Regulation of GAPDH Isoforms and Site-specific Mutants—The dependence of native regulatory GAPDH (purified from spinach chloroplasts) from the ambient redox potential in the presence of spinach Trx *f* is shown in Fig. 1*A*. In this and analogous experiments the redox poise was set by a mixture of reduced and oxidized DTT, and Trx *f* was added as a potential redox mediator between DTT and regulatory cysteines of GAPDH (27, 28). The pH of the incubation mixture (pH 7.9) reflected stromal pH during photosynthesis (31). Interestingly, although reduced DTT directly activated the NADPH-dependent activity of GAPDH to some extent (3, 8, 32), oxidized DTT (20 mM) failed to show any effect in the absence of Trx *f* even after 24-h preincubation. Trx *f*, therefore, was absolutely required for the redox titration of GAPDH activity.

The activity responses of GAPDH at varying redox potential were interpolated by a Nernst equation to estimate the standard redox potential(s) ($E_{m,7.9}$) of the regulatory disulfide(s). A reasonable good fit was obtained to a Nernst equation including one single-redox component, and an average $E_{m,7.9}$ of -353 ± 11 mV for the NADPH-dependent activity was estimated (Fig. 1*A*). The redox titration of recombinant GapB gave similar results, with an average $E_{m,7.9}$ value of -347 ± 9 mV (Fig. 1*B*). In some experiments, a second redox component was detectable in both GAPDH and GapB, but this accounted for

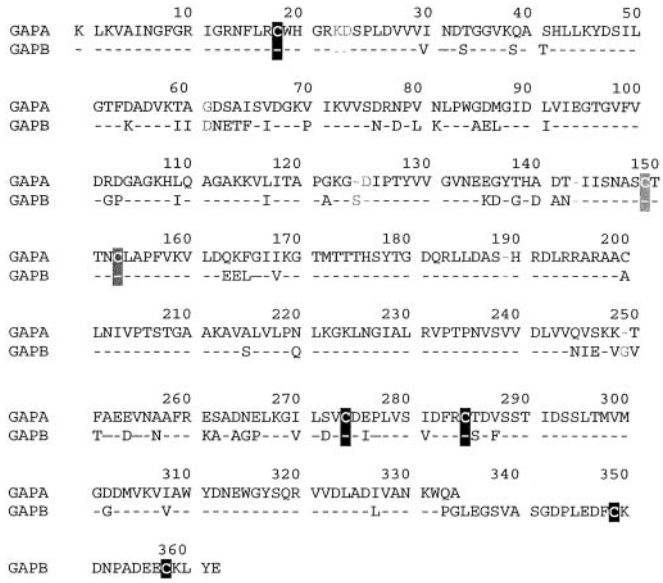

FIG. 2. **Sequence alignment of GapA and GapB from *S. oleracea*.** Residues are numbered according to *Bacillus stearothermophilus* GAPDH (35). Insertion or deletions with respect to *B. stearothermophilus* GAPDH are indicated by *gray characters* or *dashes*, respectively. In GapB, only residues different from GapA are indicated. Conserved cysteines are *highlighted*. The five Cys, which have been mutated in this work, are *white* on a *black field*; Cys-149 and Cys-153 of the catalytic site are *white* on a *gray field*.

less than 10% of the total response and was not further investigated. As expected (9), non-regulatory GapA was essentially insensitive to the redox poise (Fig. 1*B*). Therefore, the Trx-mediated regulation of the NADPH-dependent activity of native GAPDH appears to depend on GapB exclusively.

The NADPH-dependent activity of oxidized GapB ($E_h > -300$ mV) as measured by the standard assay was 60–80% inhibited with respect to the reduced form, similar to native GAPDH. Under the same conditions the NADH-dependent activity of both GAPDH and GapB, and indeed all NADH-dependent activities of various enzyme forms, were decreased by less than 20% (Fig. 1*A*, and not shown). Steady-state kinetic comparison of reduced *versus* oxidized GapB, following 3-h incubation with 20 mM reduced/oxidized DTT and 2 μg ml^{-1} Trx *f*, revealed that the inhibition of the oxidized form did not depend on decreased affinity for NADPH ($K_{m(\mathrm{NADPH})}$ 2×10^{-5} M for both forms). The GapB-oxidized form showed, instead, a decrease of ~3-fold in apparent maximum velocity ($V_{\mathrm{max(app)}}$), a kinetic parameter depending on both k_{cat} and K_m for BPGA, according to the equation (33),

$$V_{\mathrm{max(app)}} = k_{\mathrm{cat}}/(1 + K_{m(\mathrm{BPGA})}/[\mathrm{BPGA}]) \qquad \text{(Eq. 1)}$$

GapB contains seven cysteines: two are located within the CTE (Cys-349 and Cys-358) and are therefore specific for GapB; the other five (Cys-18, Cys-149, Cys-153, Cys-274, and Cys-285) are conserved also in GapA (Fig. 2). During the catalytic cycle, Cys-149 is engaged in the formation of the covalent intermediate hemithioacetal (34), and the vicinal Cys-153 is close to the active site (26) making it an unlikely candidate for the formation of a regulatory disulfide bridge.

To further characterize the redox regulatory mechanism of GAPDH, and to locate the target cysteines of Trx *f* activity, we specifically mutagenized all Cys residues of GapB into Ser, except Cys-149 and Cys-153. Also, we produced a C349S/C358S double mutant (with both CTE cysteines exchanged into Ser) and a mutant lacking the CTE altogether (GapBΔ_{CTE}). The heterologously expressed mutant proteins were purified to homogeneity and found to be enzymatically active (specific activ-

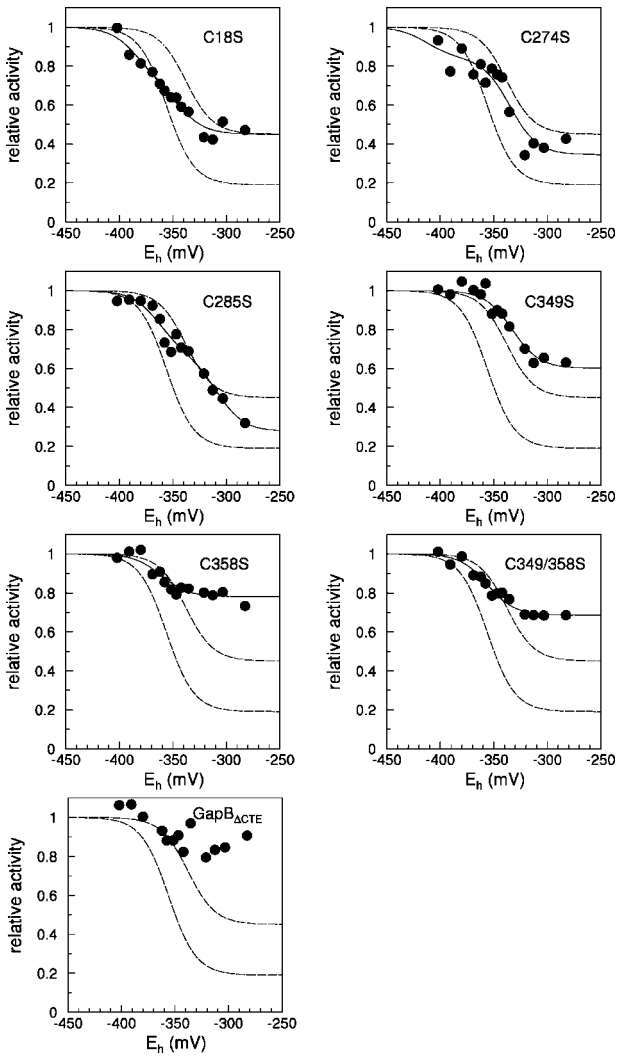

FIG. 3. **Redox titration of site-specific GAPDH mutants in the presence of thioredoxin *f*.** All the data (for each mutant as reported in each *panel*) were collected under conditions as reported in Fig. 1. Interpolation curves (*full lines*) were obtained by non-linear regression of the data using a Nernst equation with either one (C349S, C358S, C349S/C358S, or GapBΔ_{CTE}) or two redox components (C18S, C274S, or C285S). For comparison, *dashed sigmoids* in each *panel* represent limiting redox responses of GapB ($E_{m,7.9}$ −347 ± 9 mV, oxidized/reduced activity ratio 0.32 ± 0.13), *i.e.* the $E_{m,7.9}$ of the lower sigmoid is −356 mV (mean ± S.D.) and the oxidized/reduced activity ratio is 0.19 (mean-S.D.), whereas in the upper sigmoid $E_{m,7.9}$ is −338 mV and the oxidized/reduced activity ratio is 0.45. The responses of mutants C18S, C274S, and C285S were included within the two limiting sigmoids of GapB, suggesting that they were affected by the redox poise in a similar way. Other mutants (C349S, C358S, C349S/C358S, and GapBΔ_{CTE}) behaved differently, in particular their oxidized/reduced activity ratio was higher than that of GapB.

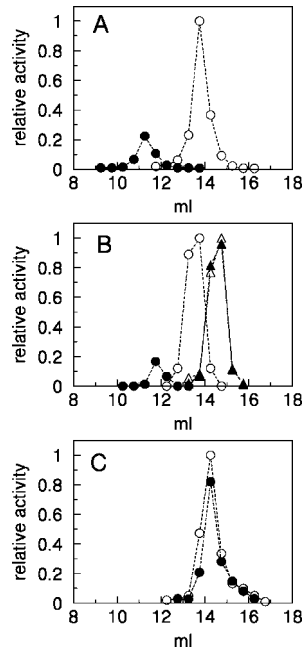

FIG. 4. **Size exclusion chromatography (Superdex 200 HR10/30, SMART System) of GAPDH isoforms and mutants under different conditions.** The column was equilibrated with 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 14 mM β-mercaptoethanol, 150 mM KCl, plus additions as indicated. Fractions of 0.5 ml were collected. All activities were measured with NADPH. *A*, native regulatory GAPDH purified from spinach chloroplasts was incubated for 1 h with 0.5 mM NADP (○) or 0.5 mM NAD (●) and then chromatographed in the presence of the corresponding coenzyme. *B*, recombinant GapB (*circles*) or GapA (*triangles*) were incubated and chromatographed in the presence of either NADP (○, GapB; △, GapA) or NAD (●, GapB; ▲, GapA). *C*, recombinant GapBΔ_{CTE} incubated as in *A* with either NADP (○) or NAD (●). Absorbance at 280 nm was recorded on-line (not shown), and GAPDH elution volumes were determined by 280-nm absorbance. Apparent molecular masses were estimated on the basis of the column calibration (Table I).

ities between 80 and 130 μmol min^{-1} mg^{-1}).

All mutants were somehow affected in their redox sensitivity (Fig. 3). The three mutants C18S, C274S, and C285S, carrying mutations on cysteines not belonging to the CTE, were quite sensitive to the redox potential. At variance from GapB, however, redox titrations of these mutants evidenced the existence of two redox components. The component accounting for most of the redox-dependent response had $E_{m,7.9}$ values close to that of GapB (C18S, −345 mV; C274S, −340 mV; and C285S, −340 mV). The second component exhibited a smaller control on the activity and was therefore difficult to define in terms of redox potential.

Mutants C349S, C358S, and GapBΔ_{CTE} and the double mutant C349S/C358S were, on the contrary, less sensitive to the redox potential than GapB (Fig. 3) suggesting an involvement of CTE cysteines in the redox regulation of GapB activity. However, whereas the mutant GapBΔ_{CTE} appeared to be almost redox-insensitive, the other mutants showed some redox dependence. Under oxidizing conditions ($E_h > -330$ mV), mutants C358S and C349S/C358S were 20% to 30% inhibited with respect to their reduced forms. Nernst interpolation of the redox-sensitive activity of these mutants gave $E_{m,7.9}$ values of −350 mV (C358S) and −355 mV (C349S/C358S). Mutant C349S was characterized by a less reducing $E_{m,7.9}$ (−330 mV) and substantial redox sensitivity (40% inhibited under oxidizing conditions).

NAD(P)-dependent Regulation of GapB Mutants and Aggregation States—It is long known that NADP activates the NADPH-dependent activity of regulatory GAPDH by stabilizing the active tetrameric conformation, whereas NAD stabilizes a less active oligomer of high molecular mass (Fig. 4) (3, 7, 8). Recombinant GapB does also aggregate to oligomers in the presence of NAD, but GapA exists only as a tetramer (12, 13, 15). The apparent molecular mass of GAPDH isoforms and mutants were estimated by size-exclusion chromatography on a Superdex 200 column calibrated with standard globular proteins (Fig. 4 and Table I). GapA eluted as a 131-kDa protein both in the presence of NAD and NADP, a value similar to the theoretical molecular mass of the tetramer (145 kDa). In the presence of NADP the GapB had an apparent molecular mass of 240 kDa, significantly higher than its calculated molecular mass as a tetramer (157 kDa). On the other hand, mutant GapBΔ_{CTE} behaved as a 149-kDa protein under same condi-

TABLE I

Apparent molecular mass and possible subunit composition of GAPDH isoforms and site-specific mutants as measured by size-exclusion chromatography on Superdex 200

Elution volumes were determined by the absorption peaks at 280 nm, and apparent molecular masses were calculated on the basis of column calibration. Samples were preincubated with either 0.5 mM NADP (*NADP* column), or 0.5 mM NAD (*NAD* column), and chromatographic runs were performed in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 14 mM β-mercaptoethanol, 150 mM KCl plus either 0.2 mM NADP or 0.2 mM NAD.

GAPDH isoform or mutant	Theoretical mol. mass (tetramer)	Apparent mol. mass (NADP)	Subunit composition	Apparent mol. mass (NAD)	Subunit composition
	kDa			*kDa*	
Regulatory GAPDH (spinach chloroplast)	151	188	A_2B_2[a]	765	A_8B_8[b]
GapA	145	131	A_4	132	A_4
GapB	157	240	B_4	540	B_8[b]
C18S	157	247	B_4	542	B_8[b]
C274S	157	240	B_4	543	B_8[b]
C285S	157	231	B_4	543	B_8[b]
C349S	157	282	B_4	264	B_4
C358S	157	254	B_4	260	B_4
C349S/C358S	157	286	B_4	253	B_4
$GapB_{\Delta CTE}$	146	149	B_4	165	B_4

[a] Based on the assumption that the apparent molecular mass of the GapA subunit was 32.7 kDa (131/4) and that of GapB was 60 kDa (240/4).
[b] A likely value: the large conformational changes caused by NAD on GapB subunits leave some uncertainty.

tions, matching its theoretical tetramer molecular mass of 146 kDa. Taken together these results suggest an extended conformation of B_4 tetramers, made bulky by the CTE. The latter, under these conditions, is probably exposed to the medium without sticking to the surface of the tetramer.

NAD induced the association of GapB into a 540-kDa form with reduced NADPH-dependent activity (Fig. 4 and Table I). Mutants C18S, C274S, and C285S behaved like GapB in this respect (Fig. 5 and Table I). As already shown (12, 13) the mutant $GapB\Delta_{CTE}$ was unable to aggregate in the presence of NAD (Fig. 4). Most remarkably, mutants C349S and C358S and the double mutant C349S/C358S also failed to change their tetrameric association state whether chromatographed in the presence of NAD or NADP (Fig. 5 and Tab. 1). Therefore, the mutation of the two cysteines of the CTE not only affected the redox regulation by Trx *f* (Fig. 3) but also caused insensitivity to the effector NAD both in terms of kinetic regulation and change of quaternary structure. This suggests that the kinetic and structural effects of NAD are mediated by the formation of a regulatory disulfide in the CTE.

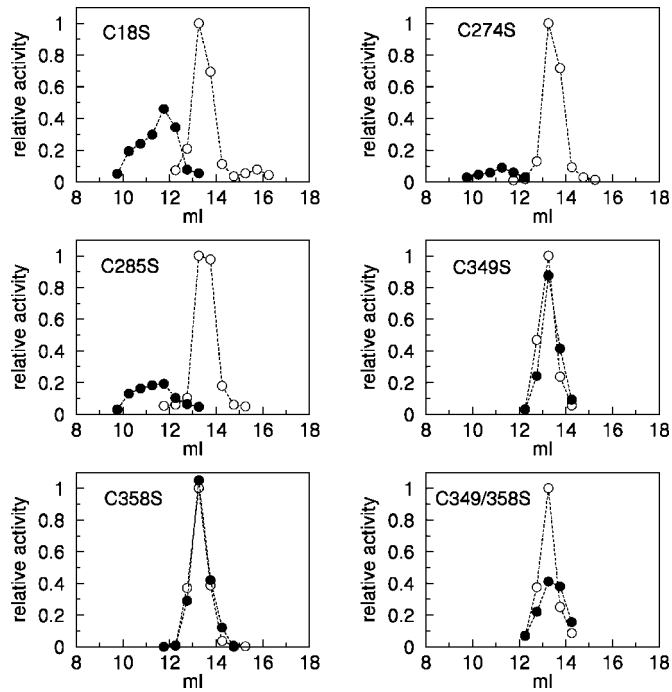

FIG. 5. **Size exclusion chromatography (Superdex 200 HR10/30, SMART System) of GapB site-specific mutants in the presence of either 0.5 mM NADP (○) or 0.5 mM NAD (●).** Each purified mutant protein was incubated with 0.5 mM NADP or NAD for 2 h before loading. The column was equilibrated with 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 14 mM β-mercaptoethanol, 150 mM KCl, plus 0.2 mM NADP or NAD. Fractions of 0.5 ml were collected and assayed for NADPH-dependent activity. Apparent molecular masses were calculated as in Fig. 4 (see Table I).

DISCUSSION

Photosynthetic GAPDH has been the first light-regulated enzyme of the Calvin cycle to be discovered (14). Thioredoxins have long been suggested to be involved in the light activation phenomenon (5, 12, 25), albeit without compelling evidence about the identity of the target GAPDH cysteines (36). The NADPH-dependent activity of purified regulatory GAPDH (A_xB_x) is regulated by the ambient redox potential through the action of Trx *f*. The standard redox potential of the spinach enzyme at the typical pH value of the stroma in the light ($E_{m,7.9}$ −353 ± 11 mV, this work) was close to the value measured for tomato leaf GAPDH ($E_{m,7.9}$ −360 mV, ref. 30) and slightly more reducing than spinach Trx *f* ($E_{m,7.9}$ −340 mV, ref. 28; or −325 mV, ref. 30), suggesting that substantial redox activation of GAPDH *in vivo* would require full reduction of the Trx *f* pool (37). However, BPGA and other activators (3, 4, 6–8) also contribute to the activation process, and this might help to explain why full activation of GAPDH in intact spinach leaves was attained at irradiance levels well below photosynthetic saturation (15).

The redox regulation of regulatory GAPDH is completely dependent on subunit B and affects the NADPH-dependent activity alone. The redox dependence of GapA and of the NAD-dependent activities of all enzyme forms amounts to <20% of the maximum catalytic capacity (Fig. 1). Redox titration analysis of GapB ($E_{m,7.9}$ −347 ± 9 mV) closely overlapped the redox titration of regulatory GAPDH, whereas the activity of GapA was virtually unaffected. It should be noted that regulatory GAPDH might be expected to be less redox-sensitive than GapB, due to the presence of redox-insensitive GapA subunits, but this was not the case: the redox sensitivity of regulatory GAPDH was the same as for GapB, or even higher. The simplest interpretation is that the functional unit of regulatory GAPDH may be constituted by a pair of GapA and GapB subunits under stringent redox control by GapB.

In performing a biochemical analysis of site-specific mutants, we have observed that the redox regulation of spinach

GapB was much decreased when Cys-358 and Cys-349 of the CTE were individually mutated into Ser. In particular, mutant C358S was almost completely redox-insensitive (similar to $GapB\Delta_{CTE}$ and GapA), whereas mutant C349S still maintained weak redox sensitivity. Other mutations, directed to all the cysteines that might potentially form a disulfide with Cys-358, did not cause any major change in redox regulation. The Cys-358/Cys-349 disulfide is therefore one main target of Trx *f* regulatory activity. In agreement with this result, the existence of a disulfide bridge between the two conserved Cys of the CTE in pea leaf GAPDH has recently been established (23). However, the residual redox sensitivity of the C349S mutant might suggest that Cys-358 could form, in the absence of Cys-349, a secondary and less effective regulatory disulfide with another Cys. The observation that the $E_{m,7.9}$ of mutant C349S (−330 mV) is different from GapB (−347 mV) is consistent with this hypothesis, although the identity of the alternative Cys is not readily apparent. A similar situation is found in pea chloroplast fructose-1,6-bisphosphate phosphatase. In this redox-regulated enzyme, a regulatory disulfide between Cys-153 and Cys-173 was detected in the crystalline-oxidized form (38). However, a secondary disulfide between Cys-153 and Cys-178 could apparently form in a mutant having Ser substituted for Cys-173 (39, 40).

In their extensive work in 1995 on the regulation of spinach GAPDH, Baalmann and coworkers (8) did not observe any dramatic change in $K_{m(BPGA)}$ or $K_{m(NADPH)}$ upon reduction of the enzyme. They suggested instead that the major effect of reduction consisted in a 10-fold decrease in the activation constant for BPGA. Here we show that the oxidation of GapB by oxidized Trx *f* also has the effect of decreasing the apparent V_{max} by a factor of 3 on average. Because apparent V_{max} is influenced by both k_{cat} and $K_{m(BPGA)}$ (33), Baalmann *et al.*'s (8) and our own results concur in supporting the conclusion that oxidation of GapB lowers the k_{cat} of the NADPH-dependent reaction. Interestingly, the specific activity of the mutants characterized by low redox sensitivity ($GapB\Delta_{CTE}$, C349S, C358S, and C349S/C358S) were all in the same range of fully activated GapB and of GapA (*i.e.* 80–130 μmol $min^{-1}mg^{-1}$), indicating that an intact CTE could only inhibit the activity, never activate it. Oxidized CTE can thus be regarded as an autoinhibitory domain, which, by lowering the k_{cat}, reduces the catalytic efficiency of the enzyme when using NADPH as the co-substrate.

It remains to be explained how the formation of the disulfide in the CTE might depress the NADPH-dependent activity without affecting the NADH-dependent one. To address this point the recent publication of the crystal structure of tetrameric GapA from spinach chloroplasts in complex with NADP may provide a clue (26). GapA monomers are basically constituted by two distinct domains: the coenzyme-binding domain and the catalytic domain. However, a long S-shaped loop (S-loop) of the catalytic domain protrudes toward the coenzyme-binding domain of the adjacent subunit, taking contact with the 2′-phosphate group of bound NADP. Because the 2′-phosphate of NADP is the hallmark to distinguish NADP(H) from NAD(H) and directly involved in enzyme activation (3), the catalytic domain could sense the bound coenzyme by means of the S-loop. Apart from the CTE, the amino acid sequences of GapA and GapB are 80% identical, and the respective structures are presumably similar. The very fact that only the NADPH-dependent activity of GapB is redox-regulated suggests that oxidized CTE might interact with the S-loop, thereby hindering the NADPH-sensing function and specifically affecting the NADP-depending activity. As already observed (41), nine CTE residues are negatively charged (Glu or Asp), and five positively charged Arg are present in the S-loop (Arg-183 to Arg-197, Fig. 2). An ionic interaction between the S-loop and the oxidized conformation of the CTE seems therefore feasible.

The NAD-induced association of GapB into high molecular weight isoforms leads to a substantial decrease of NADPH activity in the standard assay. Scagliarini *et al.* (9) showed in 1998 that inhibition of the NADPH activity of spinach GAPDH-A_8B_8 is a consequence of a 6-fold decrease in V_{max}, the other basic kinetic parameters remaining unchanged. $GapB\Delta_{CTE}$ fails to associate in the presence of NAD, nor does its activity undergo any change upon the treatment (12, 13). Interestingly, mutants C358S and C349S and the double mutant C358/349S behaved exactly like $GapB\Delta_{CTE}$, whereas the mutants C18S, C274S, and C285S behaved like GapB and regulatory GAPDH. Because NAD induces aggregation of GapB, but not of mutants unable to form a disulfide at the CTE, we conclude that the disulfide Cys-349/Cys-358 is necessary for aggregation. However, this disulfide is probably not sufficient itself to accomplish the whole process, because oxidized GapB (by oxidized Trx) was found to keep its tetrameric conformation (not shown). The association process, therefore, appears to require both NAD and the Cys-349/Cys-358 disulfide.

The regulatory mechanism of photosynthetic GAPDH nicely fit in the physiology of the chloroplast under varying photosynthetic conditions. At the onset of light, the CTE disulfide of GAPDH is reduced by the pool of Trx *f*, which is kept reduced by the activity of photosystem I, resulting in (i) increase of the k_{cat} of the NADPH-dependent reaction (this work) and (ii) reduction of the activation constant for the substrate BPGA (8). Under these conditions the concentrations of all intermediates of the Calvin cycle increase, including those of the GAPDH activators such as BPGA (16, 42, 43), thereby inducing the GAPDH-activated state and BPGA-dependent dissociation of oligomeric GAPDH into active tetramers (7, 8). In the dark, the redox-regulated Calvin cycle enzymes are oxidatively inactivated, although the nature of the oxidant is uncertain. GAPDH is efficiently inactivated by oxidized Trx *f*. Alternatively, the enzyme is inactivated and made to associate when NAD substitutes NADP at the coenzyme-binding site. Both mechanisms may be relevant *in vivo*, because the stromal NADP(H)/NAD(H) ratio in spinach leaves is known to shift from a value of 4–5 in the light to 0.6–0.7 in the dark (43). We suggest that NAD-dependent inactivation of GAPDH may be a major mechanism of dark regulation, in addition to the redox state of the thioredoxin pool.

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The C-terminal Extension of Glyceraldehyde-3-phosphate Dehydrogenase Subunit B Acts as an Autoinhibitory Domain Regulated by Thioredoxins and Nicotinamide Adenine Dinucleotide

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