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

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 ($E_{m,7.9}$: $-353 \pm 11$ mV) through the action of thioredoxin $f$. The redox dependence of recombinant GapB ($E_{m,7.9}$: $-347 \pm 9$ 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.

By GapA and GapB subunits in apparent stoichiometric ratios (1, 2). The $A$-isoform is finely regulated (3–8), whereas the $A_2$-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 concomitantly with dissociation of the high oligomers into lower molecular weight forms, mostly active tetrmers (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 ($A_4$), whereas NAD(H) does the opposite by inducing the enzyme to aggregate into a high oligomer (approximately a hexadecamer, $A_{16}$) (3, 7, 8). The inter-exchange between $A_4$ and $A_{16}$ conformations has been suggested to be part of the light-activation mechanism (15, 16), but 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 $A_4B_2$ 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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In the present study we have investigated the redox regulation of photosynthetic GAPDH by a site-specific mutagenesis
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 the light of the recent structural characterization of the α 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 GapBΔCTE, inserted in the expression vector pET-14 and kindly provided by N. Wedell, were excised from the plasmid with endonucleases BamHI and NdeI (Promega) and purified from low melting point agarose gel (0.8%, w/v) with the QIAquick gel extraction kit (Qiagen). Fragments were ligated into BamHI- and NdeI-predigested pET28a(+) (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: C185SUp: 5′-TG TCT GTA GGA ATT TCT CCA AGG ACC G-3′ and C185Down: 5′-CGT TGG GGA TGT GGC CTA AGG AAA TCA GAA CTC CTG AAA TCA ACT GAC ACG-3′; C274SUp: 5′-TGT CAG GGT TCG TAT GTC GGT CTT CA-3′; C274Down: 5′-TTT ATT GAT GAG AAA TCA GAA CTT TGG TAT GCA ACC GAC G-3′; C285SUp: 5′-TCT CAG TTA CAA CTG TCA ACA GAG GGA TCT CCA AAG TCG-3′; C285Down: 5′-ATG AAG ATT TCT CCA AAG TCG ATT TCT CAA TGG CTG-3′ and C286SUp: 5′-ATG AAG ATT TCT CCA AAG TCG ATT TCT CAA TGG CTG-3′ and C286Down: 5′-ATT GAT GAG AAA TCA GAA CTT TGG TAT GCA ACC GAC G-3′. PCR and transformation reactions were performed according to the QuikChange™ site-directed mutagenesis kit (Stratagene). Single colonies from each mutagenesis reaction were picked up, again before storage at −4°C and GAPB.

In a typical preparation, the construct containing the gene for the desired GAPDH subunit was transferred from plasmidic DNA to *E. coli* cells, strain BL21(DE3), and transformed in the presence of either Trx or Trx(−) HI-predigested pET-28a(+) (Novagen), and the ligation mixtures were used to transform *E. coli* cells, strain 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, and grown overnight at 37°C under vigorous shaking (150–170 rpm). Plasmidic DNA have been extracted and sequenced before transforming the constructs into *E. coli* cells.

**Redox Regulation of GAPDH**

Native regulatory GAPDH from spinach chloroplasts was purified according to ref. 9, except for the final size-exclusion chromatographic step, 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 MgCl₂, 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 NADP or NADH. For calculations, an ε₄₀₀ for NADPH of 6.23 μM⁻¹ cm⁻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 Titration**—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, redox mixtures were either diluted or concentrated by ultrafiltration (Centricon, YM10 membrane) to give a final NADP-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 μM of recombinant spinin Trx f, kindly provided by P. Schürmann, and 20 mM DTT in various dithioldisulfide ratios, in a total volume of 100 μl (27, 28). Following incubation, the NADPH-dependent activity (or in some cases the NAD-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 dihedral angle 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 Eₐₐₐₐ 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 μM 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 (g), the difference between the relative activity of the fully reduced form and the relative activity of the most oxidized form. After titration analysis, the exact value of this parameter (between 0 and 1) was optimized by the software.

**Size-Exclusion Chromatography**—The aggregate 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 (Gil Filtration LMW and HMW Calibration kit, Amersham Biosciences). A linear relationship between log apparent molecular masses and elution volume was obtained in the range of 13.7 (thymoglobin) to 22.3 kDa (trypsin activity was determined by the standard assay with NADP. Precise elution volumes were recorded in correspondence of the 280-nm absorbance peak, and apparent
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 denaturing 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. 1A. 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) (Eₘ,₉) of the regulatory disulfide(s). A reasonable good fit was obtained to a Nernst equation including one single-redox component, and an average Eₘ,₉ of −353 ± 11 mV for the NADPH-dependent activity was estimated (Fig. 1A). The redox titration of recombinant GapB gave similar results, with an average Eₘ,₉ value of −347 ± 9 mV (Fig. 1B). In some experiments, a second redox component was detectable in both GAPDH and GapB, but this accounted for 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. 1B). 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 (Eₘ > −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 GapA and GapB, and indeed all NADH-dependent activities of various enzyme forms, were decreased by less than 20% (Fig. 1A, 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 (Kₛₐₙ/NADPH: 2 × 10⁻⁵ M for both forms). The GapB-oxidized form showed, instead, a decrease of −3-fold in apparent maximum velocity (Vₘₐₓ/app), a kinetic parameter depending on both kₑₐₜ and Kₘ for BPGA, according to the equation (33),

\[ V_{\text{max/app}} = k_{\text{cat}}(1 + K_m/\text{BPGA})/\text{BPGA} \]  

(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 (GapBΔCTE). 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 GapBΔCTE) or two redox components (C18S, C274S, or C285S). For comparison, dashed sigmoid in each panel represent limiting redox responses of GapB (Em(7.9)≈347±9 mV, oxidized/reduced activity ratio 0.32±0.13), i.e. the Em(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 Em(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 sigmoid of GapB, suggesting that they were affected by the redox poise in a similar way. Other mutants (C349S, C358S, C349S/C358S, and GapBΔCTE) behaved differently, in particular their oxidized/reduced activity ratio was higher than that of GapB.

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 Em(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 GapBΔCTE 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 GapBΔCTE appeared to be almost redox-insensitive, the other mutants showed some redox dependence. Under oxidizing conditions (Em ≈−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 Em(7.9) values of −350 mV (C358S) and −355 mV (C349S/C358S). Mutant C349S was characterized by a less reducing Em(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 under similar conditions.

Redox Regulation of GAPDH

44949

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 GapBΔCTE incubated as in A with either NADP (○) or NAD (●). Absorbance was monitored at 280 nm, peak absorbance was denoted as 100% (not shown). Absolute elution volumes were determined by 280-nm absorbance. Apparent molecular masses were estimated on the basis of the column calibration (Table I).
performed in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 14 mM reducing than spinach Trx enzyme at the typical pH value of the stroma in the light (Fig. 3) but also caused insensitivity of either 0.5 mM NADP (A) or 0.5 mM NAD (B). Fractions of 0.5 ml were collected and assayed for NADPH-dependent activity. Apparent molecular masses were calculated as in Fig. 3 (see Table I).

Amberley and smart 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 NADP-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 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

### Table I

| GAPDH isoform or mutant | Theoretical mol. mass (tetramer) | Apparent mol. mass (NAD) | Subunit composition | Apparent mol. mass (NAD) | Subunit composition |
|-------------------------|---------------------------------|-------------------------|---------------------|-------------------------|---------------------|
| Regulatory GAPDH        | 151                             | 188                     | A_{4}B_{4}           | 765                     | A_{4}B_{4}           |
| (spinach chloroplast)   |                                 |                         |                     |                         |                     |
| GapA                    | 145                             | 131                     | A_{4}              | 132                    | A_{4}              |
| GapB                    | 157                             | 240                     | B_{4}              | 540                    | B_{4}              |
| C18S                    | 157                             | 247                     | B_{4}              | 542                    | B_{4}              |
| C274S                   | 157                             | 240                     | B_{4}              | 543                    | B_{4}              |
| C285S                   | 157                             | 231                     | B_{4}              | 543                    | B_{4}              |
| 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_{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.

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_{4}B_{4}) 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 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 NADP-dependent activities of all enzyme forms amounts to <20% of the maximum catalytic capacity (Fig. 1). Redox titration analysis...
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 substituted for Cys-173 (39, 40). Cys-178 could apparently form in a mutant having Ser substitution (38). However, a secondary 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 also has the effect of decreasing the apparent $V_{\text{max}}$ by a factor of 3 on average. Because apparent $V_{\text{max}}$ is influenced by both $K_{m}$,BPGA, and our own results concur in supporting the conclusion that oxidation of GapB lowers the $k_{\text{cat}}$ of the NADP-dependent reaction. Interestingly, the specific activity of the mutants characterized by low redox sensitivity (GapB$_{\text{CTE}}$, 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 $k_{\text{cat}}$, reduces the catalytic efficiency of the enzyme when using NADPH as the co-substrate.

It remains to be explained how the formation of the disulphide in the CTE might depress the NADP-dependent activity without affecting the NADP-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 NADP-dependent activity of GapB is redox-regulated suggests that oxidized CTE might interact with the S-loop, thereby hindering the NADP-sensing function and specifically affecting the NADP-dependent 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 NADP activity in the standard assay. Scaglìarini et al. (9) showed in 1996 that inhibition of the NADP activity of spinach GAPDH-A$_{\text{CTE}}$B$_{\text{CTE}}$ is a consequence of a 6-fold decrease in $V_{\text{max}}$, the other basic kinetic parameters remaining unchanged. GapB$_{\text{CTE}}$ 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/C349S behaved exactly like GapB$_{\text{CTE}}$, 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 fits in the physiology of the chloroplast under varying photosynthetic conditions. At the onset of light, the CTE disulfide of GapB is reduced by the pool of Trx, which is kept reduced by the activity of photosystem I, resulting in (i) increase of the $k_{\text{cat}}$ of the NADP-dependent reaction (this work) and (ii) decrease 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 NADP-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. 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 in the light to 0.6–0.7 in the dark (44). 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.

Acknowledgments—We thank Mirko Zaffagnini for experimental support. Norbert Wedell and Peter Schürmann are greatly acknowledged for providing GAPDH cDNAs and recombinant Trx, respectively.

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Redox Regulation of GAPDH

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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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J. Biol. Chem. 2002, 277:44946-44952.
doi: 10.1074/jbc.M206873200 originally published online September 20, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M206873200

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