Identification of the Cysteine Residues Involved in Redox Modification of Plant Plastidic Glucose-6-phosphate Dehydrogenase*

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The cDNA sequences encoding cytosolic and light-modulated plastidic glucose-6-phosphate dehydrogenase (G6PDH) from potato were modified by polymerase chain reaction and subsequently overexpressed in Escherichia coli. Characterization of the recombinant enzymes showed that they closely resembled their native counterparts. Treatment with reduced dithiothreitol or glutathione led to inactivation of plastidic G6PDH, whereas the activity of the cytosolic isoenzyme was not influenced by reduction. As for the native enzyme, inactivation of recombinant plastidic G6PDH was accelerated by thioredoxin and could be fully reversed by subsequent addition of oxidant. To identify the residues which are involved in redox regulation of plastidic G6PDH, each of the six cysteines in the mature protein sequence was exchanged separately for serine by site-directed mutagenesis. Two mutant proteins exhibited characteristics of the reduced wild-type enzyme. Exchange of either Cys149 or Cys157 to serine abolished the regulatory properties, suggesting that these cysteine residues are the sites responsible for redox-mediated inactivation of plastidic G6PDH.

G6PDH1 (EC 1.1.1.49) catalyzes the first step of the oxidative pentose-phosphate pathway. The main function of the enzyme is to provide NADPH for reductive biosyntheses. In plant tissues, at least two G6PDH isoforms exist in two different compartments, one in the cytosol and one in the chloroplast stroma (1, 2).

The activity of several chloroplast enzymes is known to be regulated by reversible thiol-disulfide interchange (3). During photosynthetic electron transport in the light, covalent redox modification mediated by a redox chain (the ferredoxin-thioredoxin system) leads to reductive light activation of several enzymes (4). In contrast, plastidic G6PDH is inactive in the light (5) or by reductants (6, 7) and is therefore active in the oxidized state. This regulation prevents futile cycling, i.e. simultaneous carbohydrate synthesis in the Calvin cycle and catabolism by the oxidative pentose-phosphate pathway. Thus, in accordance with its physiological role in chloroplasts, G6PDH is active only during the dark phase, when NADPH supply by the photosynthetic electron flow ceases.

Recently, we isolated cDNA sequences encoding cytosolic and plastidic G6PDH from potato (8, 9). Both plant isoforms contain six cysteine residues, but none of them at conserved positions. Notably, in the plastidic sequence all cysteines are located within a relatively short amino-terminal stretch of about 100 amino acids (9) within the NAPD binding domain (10). Comparison of the deduced amino acid sequences with those of the redox-modulated G6PDH from cyanobacteria (11–13) revealed substantial differences in the primary structures. The cyanobacterial G6PDH sequences contain two conserved cysteines at completely different positions compared with plastidic G6PDH.

To locate the cysteine residues involved in redox regulation of the chloroplast enzyme, both recombinant plant isoforms and six mutants of plastidic G6PDH from potato were expressed in Escherichia coli and characterized with respect to inactivation that can be achieved by preincubation with reduced dithiothreitol (DTTred) in vitro (6). The data show that only the plastidic enzyme is regulated by redox modification and that two of the six cysteines are involved in this mechanism. The results are discussed based on recent crystallographic data obtained with the Leuconostoc enzyme (10).

EXPERIMENTAL PROCEDURES

Materials

All biochemicals were of highest purity and purchased from Boehringer (Mannheim, FRG), Sigma (Deisenhofen, FRG), or Biomol (Hamburg, FRG). Restriction endonucleases and DNA-modifying enzymes were obtained through Boehringer (Mannheim, FRG), Life Technologies, Inc. (Eggenheim, FRG), New England Biolabs (Schwalbach, FRG) or MBI Fermentas (St. Leon Rot, FRG). Oligonucleotides for sequencing or site-directed mutagenesis were purchased either from Eurogentec (Seraing, Belgium) or MWG Biotech (Ebersberg, FRG).

Strains and Media

E. coli strain XL1-Blue served as standard host for cloning in pBlue-script II SK (pBSK), or preparation of single-strand DNA in combination with helper phage R408 (Stratagene, Heidelberg, FRG). E. coli strain BL21 (DE3) pLysS was used for overexpression of wild-type and mutant g6pdh cDNA sequences in pET11b (Novagen/AGS, Heidelberg, FRG). In addition, G6PDH-deficient E. coli strain SU294 (14) was modified for mutant analysis. To allow for expression of the pET-g6pdh constructs, SU294 was first transformed with plasmid pGPI-2 (15), a pACYC derivative, carrying the T7 RNA-polymerase gene under control of a heat-inducible lacUV5 promoter.

E. coli strains were grown according to standard procedures (16) in media containing the following antibiotics: for XL1-Blue, 10 µg/ml tetracycline; for BL21 (DE3) carrying pLysS, 25 µg/ml chloramphenicol; for strains transformed with pBSK- or pET-derivatives additionally 200 µg/ml ampicillin; and for SU294 carrying pGPI-2, 25 µg/ml kanamycin.

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1 The abbreviations used are: G6PDH, glucose-6-phosphate dehydrogenase; DTTred, reduced dithiothreitol; PCR, polymerase chain reaction; bp, base pair(s).
Oligonucleotides and Primers

Oligonucleotides were designed according to conserved regions in the cytosolic and plastidic g6pdh-cDNA sequences from potato. Primers PFL038 and PFL046 have been described previously (9). Phosphorylation of the oligonucleotides was according to standard procedures (16).

PFL070—"Sense" primer for modification of plastidic g6pdh introducing two 5'-restriction sites (Xhol underlined, BamHI in bold), corresponding to 3'-SSGFPPLA in plastidic G6PDH; 38-mer, 5'-CGGTGGAT-CC TCA AAT GGG TTT CCA ATT GC-3'.
PFL071—"Sense" primer for modification of cytosolic g6pdh introducing two 5'-restriction sites (XhoI underlined, XhoI in bold), corresponding to 3'-AAAMWCI in cytosolic G6PDH; 36-mer, 5'-GCGCTGAGAT-CC TCA AAT GGG TTT CCA ATT GC-3'.
PFL072—Degenerate sequencing primer ("antisense") based on conserved region 152-432YVEKPGG in plastidic and cytosolic potato G6PDH, respectively; 17-mer, 5'-CCA(A/G) AAN GC(T/C) TT(C/T) TCN AC-3'.
PFL073—Degenerate sequencing primer ("sense") based on conserved region 1004-398GDIAKK in plastidic and cytosolic potato G6PDH, respectively; 17-mer, 5'-GGN GA(C/T) (C/T)TN GCN AA(A/G) AA-3'.
PFL085-2—"Antisense" primer for mutagenesis of plastidic g6pdh by PCR, corresponding to 165-571CRIDKREN in plastidic and cytosolic potato G6PDH; 9-mer, 5'-GAT ACG GCT TCT CAA G-3'.
PFL085-3—"Sense" primer for mutagenesis of cytosolic g6pdh by PCR, corresponding to 165-571CRIDKREN in plastidic and cytosolic potato G6PDH; 9-mer, 5'-GAT ACG GCT TCT CAA G-3'.

All DNA-cloning techniques followed previously described standard methods (16). For overexpression of plastidic g6pdh, the 380-bp vector fragment (XhoI-D-D-AspRI) recovered from the gel and ligated to the resulting vector fragment (pET-K4His) was transformed into E. coli strain BL21 (DE3) preloaded with 50 mM CuSO4.

Modification of Plastidic g6pdh cDNA—All DNA-cloning techniques followed previously described standard methods (16). For overexpression of plastidic g6pdh with 10 N-terminal histidine residues ("His-tag"), clone pBSK-4.3 carrying the full-length cDNA coding for plastidic G6PDH was digested with restriction enzymes XhoI and BamEII. The resulting vector fragment (Δ300 bp) was isolated upon agarose-gel electrophoresis. The same clone served as template for PCR (to introduce XhoI and BamHI restriction sites at the 5'-end) using sense primer PFL070 which corresponds to the deduced mature N terminus of plastidic G6PDH, and internal antisense primer PFL046 which is specific for the plastidic isoform. PCR was conducted as described previously (9), and the resulting 940-bp product was digested with XhoI and BamEII. The resulting 120-bp fragment was recovered from the gel and ligated to the Δ300-bp vector fragment. From this construct the tailored cDNA fragment was excised with BamHI and inserted into expression vector pET-16b. For overexpression, the final His-tag construct (pET-4.3His) was transformed into E. coli strains BL21 (DE3) pLysS and SU294 pGPN-2.

Modification of Cytosolic g6pdh cDNA—The cloning strategy for overexpression of cytosolic g6pdh was similar to the one described above for the plastidic isoform. Plasmid pBSK-4.3 carrying the full-length cDNA coding for plastidic G6PDH (8) was digested to completion with XhoI and partially with EcoRI. The resulting vector fragment (Δ400 bp) was gel- purified. From the same clone PCR was conducted with primers PFL071 which corresponds to the deduced mature N terminus of plastidic G6PDH, and internal antisense primer PFL046 which is specific for the plastidic isoform. PCR was conducted as described previously (9), and the resulting 940-bp product was digested with XhoI and BamEII. The resulting 120-bp fragment was recovered from the gel and ligated to the Δ300-bp vector fragment. From this construct the modified cDNA fragment was excised with XhoI and inserted into expression vector pET-16b. For overexpression, the final His-tag construct (pET-4.3His) was transformed into E. coli strains BL21 (DE3) pLysS and SU294 pGPN-2.

Site-directed Mutagenesis

Site-directed mutagenesis of the cysteine codons was conducted with the Sculptor™ in vitro mutagenesis system kit (Amersham Buchler/USB, Braunschweig, FRG) based on the phosphorothioate method (17, 18). To obtain a construct that would allow for both mobilization of single-strand DNA and g6pdh overexpression, plasmid pBSK-4.3 was modified as follows. The T7-promoter region of pBSK was deleted by HindIII and partial PvuII digestion, and the resulting vector fragment (∼163 bp) was subsequently ligated to the 5’-HindIII/EcoRI fragment of pET-4.3His. This construct was digested with BamHI (partial) and AspI (complete) and ligated to the 5’-BamHI/AspI fragment of pET-4.3His to allow transcription from the T7 promoter in pET-16b. The resulting construct pBSK-Mut was used for mobilizing single-strand DNA for site-directed mutagenesis. From this construct, however, expression levels were too low for measuring G6PDH activity. After site-directed mutagenesis, the 620-bp BstEII/ApaI fragment comprising the base substitutions were therefore reintroduced into pET-4.3His.

The plastidic sequence comprising Cys149 and Cys157 was engineered into pET-K4His by PCR using oligonucleotides PFL085-2/-3, and the QuikChange™ site-directed mutagenesis kit (Stratagene, Heidelberg, FRG). The identity of the DNA fragments was confirmed by sequence analysis after each cloning step.

DNA Sequence Analysis

Sequencing reactions based on the dideoxynucleotide chain termination method (19) were conducted with purified plasmid DNA (QIAPrep spin plasmid miniprep kit, Qiagen, Hilden, FRG) using the Sequenase Quick-Denaturase plasmid sequencing kit and 32P-labeled dATP (USB/Amersham, Braunschweig, FRG). Degenerate g6pdh-specific primers PFL072 and PFL073 were used at 10 pmol/μl of plasmid DNA.

Induction of Gene Expression and Preparation of Protein Extracts

For the synthesis of recombinant proteins, E. coli strains were grown at 37 °C in YT medium containing the appropriate antibiotics. At an optical density (A600) of 0.2, isopropyl-β-D-thiogalactoside was added to 0.5 mM final concentration. After further incubation at 37 °C for 3 h the cells were harvested by centrifugation (5,000 × g, 4 °C, 10 min), resuspended in 0.1 of the original culture volume 100 mM Tris-maleate, 0.1 mM NADP, pH 5, and subjected to two quick freeze/thaw cycles in liquid nitrogen. To disrupt chromosomal DNA, the cell suspension was sonified twice for 30 s on ice. After 10-min centrifugation in a cooled table-top centrifuge (14,000 rpm), the supernatant (crude extract) was used for determining G6PDH activity.

Protein Determination and SDS-Polyacrylamide Gel Electrophoresis

Estimation of protein concentrations was according to Bradford (20) using bovine serum albumin as reference protein. For SDS-polyacrylamide gel electrophoresis, proteins were separated in 12% SDS-polyacrylamide gels (21) and stained with Coomassie Brilliant Blue. Dalton Mark VII-L (Sigma, Deisenhofen, FRG) served as a molecular mass standard.

Metal Chelate Chromatography

Purification of recombinant His-tag proteins on various matrices (Chelating Sepharose, Pharmacia, Freiburg, FRG; Ni-NTA resin, Qia-gen, Hilden, FRG; TALON™ metal affinity resin, CLONTECH, Palo Alto, CA) followed the CLONTECH protocol supplied for the use of TALON™ resin. As an alternative to NiSO4, Chelating Sepharose was preloaded with 50 mM CuSO4.

G6PDH Enzyme Activity Assay

G6PDH activity was measured at 340 nm in a double-wavelength spectrophotometer (Sigma-Eppendorf ZFP22, Berlin, FRG). Standard G6PDH assays were performed as described previously (8). Metal-dependent inactivation of G6PDH was assayed in the presence of 0.5–4 mM of either CuCl2, CuSO4, NiSO4, or ZnSO4.

Incubation with Thiols

All experiments were performed at room temperature under nitrogen atmosphere. Solutions containing thiols were prepared freshly with degassed buffer (100 mM Tris-HCl) and adjusted to pH 8. The standard inactivation assay contained the sample and DTTred final concentrations (62.5 mM) in a total volume of 40 μl was incubated for 10 min at room temperature prior to measuring enzyme activity.

To examine the time dependence of reduced enzyme inactivation, preincubation was performed in a larger volume and aliquots were removed after different time intervals. For reactivation of the reduced enzyme, samples were diluted with the same volume of GSSG or sodium tetra-thionate in 100 mM Tris-HCl, pH 8, yielding a twofold molar excess over GSH or DTTred, respectively, prior to measuring G6PDH activity. The corresponding controls were diluted with buffer alone.
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RESULTS

Since initial expression of plastidic G6PDH as a glutathione S-transferase fusion protein led to barely detectable enzyme activity (9), the two plant G6PDH isoforms were overexpressed as amino-terminal His-tag proteins in E. coli strain BL21. Both exhibited measurable G6PDH activity in crude bacterial extracts that was stable at room temperature for several hours. Without addition of substrate (Glc-6-P) no enzyme activity was detected. The majority of plastidic G6PDH, however, accumulated in inclusion bodies, while most of the cytosolic isoenzyme remained soluble. Attempts to purify the recombinant enzymes by metal-chelate chromatography via their amino-terminal His-tags under native conditions yielded electrophoretically pure preparations (data not shown), but resulted in complete inactivation of both isoenzymes. This was observed for Ni²⁺ and Cu²⁺ immobilized on chelating Sepharose (Pharmacia, Heidelberg, FRG), and also with commercially available matrices (TALON™ resin, CLONTech, Heidelberg, FRG; NiNTA resin, Qiagen, Hilden, FRG). Since the presence of different divergent metal cations in the standard test inhibited G6PDH activity (Zn²⁺>Ni²⁺>Co²⁺>Cu²⁺), and neither addition of EDTA nor desalting reconstituted activity after purification (data not shown), we suspect that inactivation of the His-tagged G6PDH isoforms occurs early in purification and is due to a detrimental effect of the metal cations. The properties of the recombinant enzymes were therefore assessed in crude bacterial extracts. Although induction levels varied between different experiments, G6PDH activities were clearly detectable (at least 70% of the total activity in BL21) and are given in relative units (expressed as percent of control).

First, the effect of different reducing agents on the activity of both G6PDH isoenzymes was analyzed. Inactivation by reduced thiols was seen only for the plastidic isofrom. The activity of the cytosolic enzyme assayed in parallel was not influenced (Fig. 1). The rate of inactivation of the plastidic isofrom with either DTTred or GSH was dependent on reductant concentration (Figs. 1 and 2), and could be completely reversed by addition of oxidant (exemplarily shown in Fig. 2 for GSH/GSSG) which shows that the enzyme was not irreversibly modified.

In chloroplasts, thioredoxins are known to mediate redox modification of stromal target enzymes. When recombinant spinach thioredoxin was included during preincubation with reductant, inactivation of the plastidic enzyme was markedly accelerated. This effect was specific for thioredoxin m and not observed with thioredoxin f. The activity of the cytosolic enzyme was not influenced by either thioredoxin species (data not shown).

Comparison of the cysteine positions in cytosolic, plastidic, and cyanobacterial G6PDH sequences did not indicate which of the six residues in the mature plastidic enzyme might be involved in redox regulation (see Fig. 5). Therefore, each of the six cysteine codons in the plastidic cDNA sequence from potato was exchanged for serine by 1-bp substitutions. In addition, to avoid problems in the interpretation of the mutagenesis effects, the G6PDH-deficient E. coli strain SU294 (14) was engineered for the expression of wild-type and mutant constructs. Although lower than in BL21, expression levels of the recombinant plant isoforms in SU294 sufficed for G6PDH activity measurements. In control extracts from either uninduced bacteria or induced cells lacking the recombinant pET-vector constructs, no G6PDH activity was detected. Two of the six mutant proteins (C149S and C157S) behaved differently compared with the wild type in the standard test. In the presence of DTTred, G6PDH activity of the wild type dropped to about 10% of the control samples incubated without reductant. In contrast, the activity of C149S and C157S was hardly influenced by DTTred incubation, while the other mutants behaved more or less like wild type.

The enzyme activities were compared at different substrate concentrations (ranging from 1 to 100 mM Glc-6-P). DTTred incubation of the recombinant wild-type and four mutant enzymes (C119S, C168S, C194S, and C216S) resulted in strongly decreased G6PDH activity under limiting, but not under saturating substrate concentrations (Fig. 3). In contrast, reduction had almost no effect on the activity of mutants C149S and C157S, indicating that the cysteine-to-serine substitution abolished redox regulation. In all cases, maximal catalytic velocities (Vmax) were reached at high Glc-6-P concentrations (50–100 mM).

To compare the changed substrate affinities of the oxidized and reduced states, apparent Km values of wild-type and mutant enzymes were estimated from Glc-6-P saturation curves (Table I). The recombinant wild-type enzyme shows nearly identical substrate saturation kinetics compared with the native enzyme from spinach (26). The apparent Km of the oxidized wild-type enzyme lies around 1 mM Glu-6-P and is increased...
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30–40-fold upon reduction. Four mutant enzymes, i.e., C119S, C168S, C194S, and C216S, show comparable $K_m$ shifts upon reduction. In contrast, the apparent $K_m$ values of both the oxidized and reduced states of mutants C149S and C157S lie between 20 and 30 mM, i.e. within the range of the values determined for the reduced wild-type enzyme. Compared with data obtained with the native chloroplast enzyme from pea (26), no $K_m$ shift for the coenzyme NADP (50 $\mu$M) was seen with the recombinant wild-type or the mutant proteins (data not shown).

Modeling of the three-dimensional structure of plastidic G6PDH was based on a comparison of the deduced amino acid sequence (9) with the crystallographic coordinates of the Leuconostoc enzyme (10). Fig. 4 shows the resulting model of plastid potato G6PDH as a dimer. Calculation of molecule distances reveals that amino acids Cys149 and Cys157 are the only potential candidates for disulfide-bond formation. The two cysteine residues appear to be located in an exposed loop on the surface of the protein, and thus would be freely accessible for interaction with thioredoxin. The distance between the sulfur groups is approximately 0.4 nm which is close to the theoretical value necessary for disulfide bridge formation (maximum, 0.35 nm).

To test whether the presence of the loop containing the two identified cysteine residues has an influence on the activity of the cytosolic isoenzyme, region 149CRIDKRENRCys157 in plastidic G6PDH was introduced into the cytosolic isoform by PCR, replacing the orthologous sequence 89QGKEN (for amino acid alignment, see von Schaewen et al. (9)). However, this modification did not affect the activity of the cytosolic enzyme in the presence or absence of reductant (data not shown).

**DISCUSSION**

Site-directed mutagenesis is a classical approach to examine structure-function relationships of recombinant enzymes. Using this technique, the responsible cysteine residues in NADP-malate dehydrogenase (27) and FBPase (28), two target enzymes of the ferredoxin-thioredoxin system of higher plants, have been addressed to date. Here we show which cysteines are involved in redox regulation of plastidic G6PDH. This protein represents a unique case, since in contrast to all other known redox-regulated enzymes, plastidic G6PDH is inactivated by reduction (5, 7).

Recently, we elucidated the cDNA sequences coding for cytosolic and plastidic G6PDH from potato (8, 9). Both isoforms contain six cysteine residues in their deduced amino acid sequences (Fig. 5). All cysteine residues in cytosolic and plastidic G6PDH proteins (from four different plants and one green alga), are located at conserved positions within the respective isoform groups. Therefore, experiments were performed with both recombinant G6PDH isoenzymes from potato. We could show that only plastidic G6PDH is inactivated upon reduction in vitro. Redox regulation of the recombinant cytosolic isoform, as described for the pea enzyme (29–32), could not be confirmed. Inactivation of recombinant plastidic G6PDH is fully reversible by reoxidation (Fig. 2) and thus is not due to an irreversible loss of activity. The stimulatory effect of thior-
doxin on the rate of reductive inactivation of chloroplast G6PDH has already been described (7), and was also found for the recombinant enzyme (data not shown). As has been determined for the enzyme in pea chloroplasts (26), the $K_m$ for Glc-6-P was increased from 1 mM in the oxidized state to 30–40 mM in the reduced state (Table I), with the $V_{max}$ being unaffected. However, the $K_m$ shift found for the coenzyme NADP was not observed with the recombinant plastidic enzyme.

The results obtained indicate that recombinant plastidic G6PDH expressed in *E. coli* behaves like its native counterpart in planta. This was the presupposition for investigating the molecular mechanism of redox regulation of plastidic G6PDH with respect to the involvement of cysteine residues by site-directed mutagenesis. Since affinity purification led to inactive enzyme preparations, the catalytic properties of the recombinant wild-type and the mutant plastidic G6PDH enzymes were characterized in crude bacterial extracts of a G6PDH-deficient *E. coli* strain.

Replacement of two of the six cysteines by serines (Cys$^{149}$ and Cys$^{157}$) abolished the redox-regulatory properties of the enzyme. The Glc-6-P saturation curves for the reduced and oxidized enzyme forms were almost identical, and the apparent $K_m$ values were comparable to those of the reduced wild-type enzyme. As expected, the serine-for-cysteine substitutions affect substrate affinity rather than catalytic activity.

The insensitivity toward reduction by dithiothreitol indicates that the two mutant proteins C149S and C157S behave like the wild-type enzyme in the reduced, i.e. inactive state. These results are compatible with both residues being engaged in a disulfide bridge in active plastidic G6PDH. Based on the crystallographic data obtained with the *Leuconostoc* enzyme (10), computer modeling of the plastidic potato sequence revealed that only the two experimentally identified cysteines (Cys$^{149}$ and Cys$^{157}$) would be close enough to form a disulfide bridge (Fig. 4). The two cysteine residues reside in the amino-terminal domain of the enzyme which also contains the NADP-binding site. The $K_m$ values for the coenzyme, however, were unaffected by the mutations. A similar result was obtained upon mutation of a conserved lysine residue in the coenzyme binding domain of the *Leuconostoc* enzyme which caused ineffective turnover of the substrate Glc-6-P (10). Since Glc-6-P binding occurs in the other domain of the subunit, we speculate that absence of the disulfide bridge results in destabilization, and thereby prevents either efficient binding of the substrate or sufficient proximity of the Glc-6-P and NADP binding domains.

Introduction of the loop of the plastidic isofrom comprising the two identified cysteines into cytosolic G6PDH did not affect the activity of the enzyme under reducing or oxidizing conditions (data not shown). This result emphasizes the structural differences that must exist between the two plant isoforms, since polyclonal antisera raised against the recombinant proteins recognize specifically only their cognate G6PDH in blotting experiments (9).

In conclusion, the data presented suggest that the two vicinal cysteines identified in the plastidic potato sequence are necessary but not sufficient for redox regulation of G6PDH from plants.

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