Recently, we demonstrated that the control of mitochondrial redox balance and oxidative damage is one of the primary functions of mitochondrial NADP⁺-dependent isocitrate dehydrogenase (IDPm). Because cysteine residue(s) in IDPm are susceptible to inactivation by a number of thiol-modifying reagents, we hypothesized that IDPm is likely a target for regulation by an oxidative mechanism, specifically glutathionylation. Oxidized glutathione led to enzyme inactivation with simultaneous formation of a mixed disulfide between glutathione and the cysteine residue(s) in IDPm, which was detected by immunoblotting with anti-IDPm IgG. The inactivated IDPm was reactivated enzymatically by glutaredoxin2 in the presence of anti-GSH IgG. The inactivated form of IDPm is a glutathionyl mixed disulfide. Mass spectrometry and site-directed mutagenesis further confirmed that glutathionylation occurs to a Cys²⁶⁹ of IDPm. The glutathionylated IDPm appeared to be significantly less susceptible than native protein to peptide fragmentation by reactive oxygen species and proteolytic digestion, suggesting that glutathionylation plays a protective role presumably through the structural alterations. HEK293 cells and intact respiring mitochondria treated with oxidants inducing GSH oxidation such as H₂O₂ or diamide showed a decrease in IDPm activity and the accumulation of glutathionylated enzyme. Using immunoprecipitation with anti-IDPm IgG and immunoblotting with anti-GSH IgG, we were also able to purify and positively identify glutathionylated IDPm from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice, a model for Parkinson’s disease. The results of the current study indicate that IDPm activity appears to be modulated through enzymatic glutathionylation and deglutathionylation during oxidative stress.

The initial cellular response to oxidative stress is often a reduction in the levels of GSH, which represents the major low molecular weight antioxidant in mammalian cells, and a corresponding increase of GSSG, the oxidized form of GSH (1–3). It is well established that GSH plays a central role in the cellular defense against oxidative damage (4). Thus, the oxidation of a limited amount of GSH to GSSG can dramatically change this ratio and affect the redox status within the cell.

Regulation of Mitochondrial NADP⁺-dependent Isocitrate Dehydrogenase Activity by Glutathionylation

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Under these conditions of moderate oxidative stress, thiol groups of intracellular proteins can be modified by the reversible formation of mixed disulfides between protein thiols and low molecular mass thiols such as GSH, a process known as S-glutathionylation (5). Glutathionylation, which is reversible by the actions of the enzyme glutaredoxin (thioltransferase) (6, 7), may serve as a means of protection by preventing the irreversible oxidation of cysteine to cysteine sulfonic and sulfonic acid.

One proposed mechanism leading to protein glutathionylation in vivo is the thiol/disulfide exchange mechanism (8), which occurs when an oxidative insult changes the GSSG/GSH ratio and induces GSSG to bind to protein thiols. GSSG/GSH ratio is an indicator of the redox status of the cell, and the extent of protein glutathionylation will vary accordingly; a higher ratio will promote glutathionylation, and a lower ratio will result in deglutathionylation of glutathione (9). Therefore, the regulated formation of mixed disulfides between protein thiols and glutathione redox changes has the potential to act as a reversible switch in much the same way as phosphorylation (10). A growing list of enzymes, including glyceraldehyde-3-phosphate dehydrogenase (11), protein kinase C (12), and guanylate cyclase (13), and glucocorticoid receptors (14) are potentially influenced by the formation of protein adducts with glutathione. Also transcription factors such as c-Jun appear to be redox-regulated by mechanisms that include protein S-thiolation (10, 15), and ubiquitin-activating enzymes become glutathionylated, with a concomitant decrease in the ubiquitination pathway, when cells are exposed to oxidants (16).

The isocitrate dehydrogenases (ICDHs)¹; EC 1.1.1.41 and EC 1.1.1.42) catalyze oxidative decarboxylation of isocitrate to α-ketoglutarate and require either NAD⁺ or NADP⁺, producing NADH and NADPH, respectively (17). NADPH is an essential reducing equivalent for the regeneration of GSH by glutathione reductase and for the activity of the NADPH-dependent thioredoxin system (18, 19), and both are important in the protection of cells from oxidative damage. Therefore, ICDH may play an antioxidant role during oxidative stress. In mammals, the following three classes of ICDH isoenzymes exist: mitochondrial NAD⁺-dependent ICDH, mitochondrial NADP⁺-dependent ICDH (IDPm), and cytosolic NADP⁺-dependent ICDH (IDPc) (17). We reported recently (20) that ICDH is involved in the supply of NADPH needed for GSH production

¹ The abbreviations used are: ICDH, isocitrate dehydrogenase; IDPm, mitochondrial NADP⁺-dependent isocitrate dehydrogenase; IDPc, cytosolic NADP⁺-dependent isocitrate dehydrogenase; HNE, 4-hydroxynonenal; DTT, dithiothreitol; NEM, N-ethylmaleimide; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ANSA, 8-anilino-1-naphthalene sulfonic acid; HNE, 4-hydroxynonenal; ESI-MS, electrospray ionization mass spectrometry; Grx2, glutaredoxin2; ROS, reactive oxygen species; PD, Parkinson’s disease; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid.
against cytosolic and mitochondrial oxidative damage. Hence, the damage of IDPm may result in the perturbation of the balance between oxidants and antioxidants and subsequently lead to a pro-oxidant condition. Because cysteine residues serve as an essential role in the catalytic function of IDPm (21, 22), the highly reactive sulfhydryl groups in IDPm could be potential targets of nitric oxide, peroxynitrite, 4-hydroxynonenal (HNE), H$_2$O$_2$, and diamide. Based on the reactive nature of cysteine residue(s) in IDPm at physiological pH and the fact that sulfhydryl modification results in inactivation of enzyme, we hypothesized that IDPm is a candidate for glutathionylation—sulfhydryl modification results in inactivation of enzyme, cysteine residue(s) in IDPm at physiological pH and the fact that sulfhydryl modification results in inactivation of enzyme, we hypothesized that IDPm is a candidate for glutathionylation-mediated regulation.

In this study, we report that IDPm is inactivated by the formation of a mixed disulfide between Cys$^{389}$, the active site cysteine, and GSH, and that this inactivation is reversed not only by dithiothreitol (DTT) but also more importantly by thioredoxin transferase, a thiol/disulfide oxidoreductase that is specific for glutathionyl mixed disulfide substrates and specifically utilizes GSH as a cosubstrate. This mechanism suggests an alternative modification to the redox regulation of cysteine in IDPm and suggests a possible in vivo mechanism in the regulation of IDPm activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Iodoacetate, β-NADP$^+$, NADPH, and IDPm from pig heart and GSH, GSGG, cysteine, DT, hydrogen peroxide, chymotrypsin, Pronase, trypsin, N-ethylmaleimide (NEM), diamide, rose bengal, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and 8-anilino-1-naphthalene sulfonic acid (ANSV) were purchased from Sigma. HNE was obtained from Calbiochem. Electrothrophoresis reagents and Bio-Rad protein assay kits were purchased from Bio-Rad. Antibody against IDPm was prepared from IDPm-immunized rabbit, and the antibody was purified by protein A affinity chromatography.

**Construction of Expression Vector**—A human embryonic kidney cell line, was purchased from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate, respectively. Cells were incubated in a humidified atmosphere of 5% CO$_2$ and 95% air at 37 °C.

**Measurement of IDPm Activity**—IDPm (6.5 μg) was added to 1 ml of 40 mM Tris buffer, pH 7.4, containing NADP$^+$ (2 mM), MgCl$_2$ (2 mM), and isocitrate (5 mM). Activity of IDPm was measured by the production of NADPH at 340 nm at 25 °C (21). One unit of IDPm activity is defined as the amount of enzyme catalyzing the production of 1 mol of NADPH/min.

**Immunoblot Analysis**—Proteins were separated on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and subsequently subjected to immunoblot analysis by using appropriate antibodies. Immunoreactive antigen was then recognized by using horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham Biosciences). For glutathionylation detection, blotting was performed with a secondary antibody directed against GSH. Activity of IDPm was determined by the colorimetric assay following the colorimetric reaction of GSH with NADPH.

**Isolation of Mitochondria from Rabbit Heart**—Mitochondria obtained from rabbit heart were isolated according to the procedure described in Materials and Methods.

**Isolation of Mitochondria from Rabbit Heart**—Mitochondria obtained from rabbit heart were isolated according to the procedure described in Materials and Methods.

**Incubation of Intact Mitochondria with H$_2$O$_2$ and Diamide**—Intact mitochondria were incubated with 10 μM H$_2$O$_2$ and 10 μM diamide for 1 h at 37 °C. Mitochondria were then washed with buffer and subjected to immunoblot analysis with an antibody directed against GSH.

**Control Experiments**—Control experiments were performed in the absence of glutathione and diamide.

**Isolation of Mitochondria from Rabbit Heart**—Mitochondria obtained from rabbit heart were isolated according to the procedure described in Materials and Methods.
Science) by using immunoprecipitation and subsequently resolved by SDS-PAGE. The gels were soaked in Amplify (Amersham Biosciences) and then dried, and finally the dried gels were placed in direct contact with x-ray film. For more accurate quantitation, excised gel slices were dissolved by the NCS solubilizer (Amersham Biosciences). The resulting solutions were assayed by liquid scintillation counting.

Replicates—Unless otherwise indicated, each result described in the paper is representative of at least three separate experiments.

RESULTS

Inactivation of IDPm by GSSG—Inactivation of IDPm with GSSG at pH 7.4 at 37 °C resulted in a time- and concentration-dependent loss of enzyme activity as shown in Fig. 1, A and B. At 10 mM, activity was inhibited completely with half-maximal inhibition occurring at 0.1 mM. On the other hand, 5 mM GSH did not cause any noticeable inhibition of IDPm activity. In the resting cells, the ratio of GSH to GSSG exceeds 100, whereas in various models of oxidative stress, this ratio was reported to decrease to values between 10 and 1 (8). When IDPm was incubated with the mixtures of GSH and GSSG with the ratios between 10 and 1, 15–25% of inhibition was achieved. It has been shown that a polyclonal anti-GSH antibody is very useful for the detection of glutathionylation. When IDPm was incubated with various concentrations of GSSG and subjected to Western blot analysis with a polyclonal anti-GSH antibody, the intensity of the immunoreactive 45-kDa band was increased in a concentration-dependent manner (Fig. 1C). It has been proposed that cysteine residue(s) in IDPm could be potential targets of sulfhydryl-modifying agents (21, 22). To determine whether glutathionylated cysteine(s) in IDPm are susceptible to sulfhydryl-modifying agents, IDPm was allowed to react simultaneously with GSSG and various concentrations of NEM, diamide, and HNE, lipid peroxidation product. As shown in Fig. 2, the dose-dependent decrease of glutathionylated IDPm was observed. As shown in Fig. 3A, the addition of 5 mM DTT completely reversed inhibition, suggesting that GSSG is modifying susceptible cysteine(s) on the protein through the formation of a mixed disulfide. Grx2, a thioltransferase, is a 12-kDa mitochondrial protein that is shown to specifically reverse protein-glutathione mixed disulfides by utilizing GSH as
an electron donor. Grx2 is therefore a key component of the cellular machinery in maintaining and reversing glutathionylation of susceptible protein thiols (7). Thus it can be hypothesized that a glutathionylated IDPm could be reactivated by Grx2 in the presence of GSH. As depicted in Fig. 3A, more than 80% of the original IDPm activity was recovered by the enzyme-catalyzed disulfide exchange with Grx2 (5H9262g) in the presence of 0.5 mM GSH. However, this concentration of GSH alone had no effect on the recovery of IDPm activity. The correlation between the recovery of IDPm activity and the reduction of the level of glutathionylated IDPm, which was evaluated by Western blotting with anti-GSH antibody, was observed (Fig. 3B). To confirm further how many cysteine residues are targeted for glutathionylation, IDPm was treated with 5 mM GSSG for 1 h at 37 °C and subjected to ESI-MS. Molecular masses of unmodified and GSSG-modified (305 Da) species are labeled. B, activity of wild-type and C269S IDPm. Wild-type (closed circles) and C269S mutant (open circles) IDPm were treated with various concentrations of GSSG for 1 h at 37 °C, and the remaining activity was determined. Activities are given as a percentage of the control value. Data are presented as means ± S.D. of three separate experiments. C, accumulation of the GSS-IDPm adduct in wild-type (WT) and C269S mutant IDPm. After incubation with 10 mM GSSG for 1 h at 37 °C, wild-type and mutant IDPm were characterized by SDS-PAGE followed by immunoblotting with anti-GSH IgG.
IDPm displayed the expected molecular mass of 1042 Da, and the GSSG-treated IDPm induced the formation of a peak with a molecular mass of 1349 Da, corresponding to the addition of one glutathione molecule. The other fragments do not contain the glutathione addition. The result indicates that Cys\(^{269}\) of IDPm is a target site for glutathionylation. The C269S mutant containing a cysteine to serine mutation at position 269 lost 40% of its catalytic activity. As shown in Fig. 4, B and C, the remaining activity of the C269S mutant was not affected by GSSG, and no glutathionylated IDPm was observed with 10 mM GSSG, further confirming that Cys\(^{269}\) is a target of glutathionylation of IDPm. The total number of cysteine residues in the mammalian IDPm has been reported to be 8.2–8.8 mol/mol subunits (24). In these cysteines, Cys\(^{269}\) and Cys\(^{379}\) were regulated by NEM, and the main site of modified cysteines (21), Cys\(^{269}\), has a catalytic role, most likely in the strengthened binding of Mn\(^{2+}\) in the presence of isocitrate, whereas Cys\(^{379}\) is not essential for catalysis and the NADP\(^+\)-binding site. To determine the protective effect of substrates on the glutathionylation of IDPm, IDPm was incubated with GSSG in the presence of each substrate for 1 h at 37 °C. As shown in Fig. 5A, although the incubation of IDPm with 10 mM GSSG completely inhibited the IDPm activity, the addition of 4 mM isocitrate significantly protected IDPm from inactivation. The exclusive protective effect of isocitrate compared with NADP\(^+\) suggests that GSSG reacts more readily with the isocitrate-binding site than the NADP\(^+\)-binding site. Immunoblotting with polyclonal anti-GSH antibody to detect IDPm also indicated that only isocitrate, but not NADP\(^+\) or MnCl\(_2\), exhibited a protective effect against glutathionylation. Under the conditions of oxidative stress to cells, the reactive oxygen species (ROS) can cause oxidative damage to proteins, including fragmentation of peptide. The fragmentation of IDPm by oxidative damage was measured by the disappearance of the native IDPm band at 45 kDa in denaturing electrophoresis gels. As shown in Fig. 6A, glutathionylated IDPm was protected from peptide fragmentation caused by rose bengal/light, which generates singlet oxygen, and diamide. We also examined whether or not glutathionylated IDPm becomes less susceptible to proteolytic digestion. The results indicated that glutathionylated IDPm appeared to be significantly less susceptible than the native protein to the proteolysis by trypsin, chymotrypsin, or Pronase (Fig. 6B). It can be proposed that glutathionylation plays an important protective role in the degradation of IDPm by ROS or proteases, presumably through the structural changes that may cause IDPm to be less susceptible to the attack by ROS or proteases.

**Structural Changes in Modified IDPm—**To examine the secondary structure of the IDPm species after modification with GSSG, far UV-CD spectra of nontreated and GSSG-treated IDPm were recorded and analyzed for specific elements of secondary structure. The CD spectrum of IDPm is very similar to that of the protein after modification with 5 mM GSSG, suggesting that glutathionylated IDPm does not appreciably change the secondary structure of the protein (Fig. 7A). To reveal increases in flexibility of a partial unfolding of glutathionylated IDPm, the binding of the fluorescent probe ANSA was used to detect the accessibility of the hydrophobic regions on the protein. When IDPm was exposed to GSSG for 1 h, it bound the hydrophobic probe ANSA more efficiently than does the native protein. The representative result with GSSG is shown in Fig. 7B. In an attempt to determine the effects of glutathionylation on the conformation of IDPm, the intrinsic fluorescence of the aromatic amino acids in each of the various forms of the enzyme was determined. Native IDPm exhibited a fluorescence emission spectrum typical for tryptophan residues in proteins. Upon excitation of native IDPm at 278 nm, an emission spectrum with a maximum at 333 nm was observed. The fluorescence spectra of native and GSSG-treated IDPm, normalized to the protein content, showed that modified IDPm displays a dose-dependent decrease in the quantum yield of the emission spectra and a blue shift of the maximum emission wavelength (Fig. 7C).

**Glutathionylation of IDPm in Intact Cells and Mitochondria—**Because GSSG readily glutathionylates IDPm in vitro, we examined IDPm activity and glutathionylation in HEK293 cells, an embryonic kidney cell line, after treatment with H\(_2\)O\(_2\) or diamide. It has been reported that chemical oxidants such as H\(_2\)O\(_2\) or diamide can serve as a catalyst in promoting the formation of protein-mixed disulfides with glutathione (26). As shown in Fig. 8A, a concentration-dependent decrease of IDPm activity in H\(_2\)O\(_2\)- or diamide-treated cells was observed. Mitochondrial fractions from both control and H\(_2\)O\(_2\)- or diamide-treated cells were subjected to immunoprecipitation with anti-IDPm antibody followed by separation by SDS-PAGE. Western blot analysis of purified IDPm with anti-GSH IgG revealed a concentration-dependent increase of immunoreactive bands in H\(_2\)O\(_2\)- or diamide-treated cells, whereas no increase was found in the control cells (Fig. 8B). The control incubation of cell homogenates with [\(^3\)H]GSH did not yield any significant amounts of protein-bound radiolabeled GSH. Furthermore, the purified IDPm from cell homogenates treated with GSH were analyzed by mass spectrometry, and no generation of the glutathionylated products was observed. These results confirm that artifactual intrapreparative glutathionylation has not occurred. To evaluate the reversibility of glutathionylation in mitochondria, intact respiring mitochondria were treated with 50 μM H\(_2\)O\(_2\) or 20 μM diamide for 10 min, which caused a 40–50% decline in IDPm activity. Mitochondria were solubilized and treated with DTT or Grx2/GSH. The DTT or Grx2 system was capable of reactivating IDPm inactivated by...
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FIG. 7. Structural changes in glutathionylated IDPm. A, analysis of glutathionylated IDPm by CD. Untreated (thick line) and IDPm treated with 5 mM GSSG for 1 h at 37 °C (thin line) were analyzed in a spectropolarimeter. Spectra were recorded from 190 to 250 nm, and mean residue ellipticity is plotted as a function of wavelength. B, spectrofluorometric analysis of ANSA binding to the glutathionylated IDPm. Emission spectra from 400 to 600 nm (excitation, 370 nm) of ANSA (100 μM) bound to native IDPm (lower trace) and IDPm treated with 5 and 10 mM GSSG for 1 h at 37 °C (middle and upper traces, respectively). The increase in fluorescence intensity at 490 nm resulting from the binding of ANSA to the enzyme was determined by subtracting the emission spectrum of ANSA from that of ANSA in the presence of the different forms of the enzyme. C, steady-state emission spectra of intrinsic fluorescence of native (upper trace) and IDPm treated with 5 and 10 mM GSSG for 1 h at 37 °C (middle and lower traces, respectively) were analyzed in a spectrofluorimeter. Spectra were obtained using an excitation wavelength of 278 nm and excitation and emission slits of 5 nm.

FIG. 8. Inactivation of IDPm (A) and accumulation of glutathionylated IDPm (B) in HEK293 cells by oxidants. HEK293 cells were incubated with various concentrations of H$_2$O$_2$ or diamide for 30 min at 37 °C and were disrupted by sonication. The mitochondrial fraction was prepared, and the activity of IDPm was determined. Activities are given as a percentage of the control value. Data are presented as means ± S.D. of five separate experiments. IDPm was purified from the control and the oxidant-treated HEK293 cells by using immunoprecipitation with anti-IDPm antibody as described and then characterized by SDS-PAGE followed by immunoblotting. Purified IDPm was probed with anti-GSH IgG.

treatment of mitochondria with H$_2$O$_2$ or diamide. GSH at 0.5 mM had no effect when added in the absence of Grx2 (Fig. 9, A and B). When HEK293 cells were exposed to 2 mM H$_2$O$_2$ or 1 mM diamide for 30 min and subsequently washed with PBS, the activities IDPm in treated cells were significantly reduced and gradually recovered to near control levels during further incubation (Fig. 9, C and D).

Glutathionylation of IDPm in Vivo—Parkinson disease (PD) is a progressive neurodegenerative disorder that affects primarily the dopamine neurons projecting from the substantia nigra pars compacta to the putamen and caudate regions of the brain (30). Exposure to MPTP induces PD-like symptoms in humans and causes degeneration of dopaminergic neurons in several animal species (28). MPTP is known to generate oxidative stress that leads to formation of GSSG, which forms disulfide linkages (Pr-SSG) with cysteine residues of proteins in mitochondria (29). As shown in Fig. 10, Western blot analysis of IDPm in brains from MPTP-treated mice, which were purified by immunoprecipitation with anti-IDPm antibody, with anti-GSH IgG showed pronounced increase of glutathionylated
IDPm. Control experiments confirm that artifactual intra-preparative glutathionylation has not occurred.

**DISCUSSION**

NADPH is an essential cofactor for the regeneration of GSH, the most abundant low molecular mass thiol in most organisms, by glutathione reductase in addition to its critical role for the activity of the NADPH-dependent thioredoxin system (18, 19). IDPm is a key enzyme in cellular defense against oxidative damage by supplying NADPH in the mitochondria, needed for the regeneration of mitochondrial GSH or thioredoxin. Elevation of mitochondrial NADPH and GSH by IDPm in turn suppressed the oxidative stress and concomitant ROS-mediated damage. It is well established that mitochondrial dysfunction is directly and indirectly involved in a variety of pathological states caused by genetic mutations as well as exogenous compounds or agents (30). Mitochondrial GSH becomes critically important against ROS-mediated damage because it not only functions as a potent antioxidant but is also required for the activities of mitochondrial glutathione peroxidase and mitochondrial phospholipid hydroperoxide glutathione peroxidase (31), which removes mitochondrial peroxides. NADPH is a major source of reducing equivalents and cofactor for mitochondrial thioredoxin peroxidase family/peroxiredoxin family including peroxiredoxin III/protein SP-22 (32–34) and peroxiredoxin VAOE166 (35). Therefore, any mitochondrial NADPH producer, if present, becomes critically important for cellular defense against ROS-mediated damage. In this regard, the inactivation of IDPm may result in the disruption in regulating the mitochondrial redox balance by providing NADPH.

It has been reported that IDPm contains reduced cysteinyl residues that play an essential role for enzyme activity (21, 22). The sulfhydryl groups of IDPm are susceptible to modification with ROS, reactive nitrogen species, various oxidants, and lipid peroxidation products (36–39). In the meantime, cysteine-containing proteins are susceptible to protein S-glutathionylation, the reversible covalent addition of glutathione to cysteine residues on target proteins. In this study, we present evidence indicating that IDPm can be inhibited by reversible glutathionylation. The glutathionylation of IDPm was competed with NADPH and GSH.
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sulphydryl-modifying agents such as NEM, diamide, and HNE. Because IDPm inactivation was prevented by adding its substrate isocitrate, we conclude that GSSG-binding sites are likely to include a cysteine residue near the active site. Treatment of glutathionylated IDPm with DTT or Grx2 in the presence of GSH resulted in the recovery of IDPm activity, indicating the formation of the protein-SSG species. Grx2 containing a mitochondrial leader sequence was identified in human and mouse tissue (40, 41). Unlike other glutaredoxin isofoms, Grx2 is relatively insensitive to oxidative inactivation, making it an effective enzyme for an oxidatively dynamic environment like the mitochondria (41). Using mass spectrometry and site-directed mutagenesis results revealed that Cys269, a residue which presumably resides in the isocitrate-binding site, is a target for glutathionylation.

There are several lines of evidence obtained from the present study indicating that glutathionylation of IDPm results in structural alterations. These findings are reflected in the changes in intrinsic tryptophan fluorescence and the binding of ANSA. However, the CD spectrum and, therefore, the secondary structure content of IDPm were not altered by glutathionylation, which suggests that only subtle, not drastic, conformational changes may occur in modified protein. The lower fluorescence quantum yield demonstrates the alteration of the conformational integrity in glutathionylated IDPm. Modification of IDPm by glutathionylation may lead to a slight disruption of protein structure, which is presumably responsible for the inactivation of enzymes at least in part. Among the techniques aimed at following conformational changes of proteins, binding of the fluorescent probe ANSA has been used to detect the accessibility of the hydrophobic regions on protein upon increases in flexibility or partial unfolding. Binding can be easily monitored because it is accompanied by an increase in fluorescence associated with the transfer of the ANSA from a hydrophilic to a hydrophobic environment (42). A change in ANSA fluorescence at 490 nm in IDPm modified by glutathionylation indicates conformational changes of protein.

Mitochondria are responsible for generating the ATP required for all cellular functions, for detoxifying ROS produced via mitochondrial respiration, for controlling the cellular redox state, and for regulating cytoplasmic calcium levels as activity as the major intracellular sink for this ion. Oxidative damage to the mitochondria might interfere with all of these functions (43). To maintain mitochondrial and cellular viability, the mitochondria must respond to a dynamic redox environment. Regulation of biological activity by the reversible modification of protein thiol is a growing concept in cellular defense, such as oxidative stress, the inactivation of key antioxidant enzymes would further deteriorate cell homeostasis. Therefore, glutathionylation of IDPm could be considered an adaptation of the cell to severe oxidative stress.

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The ready formation of glutathione mixed disulfide on IDPm will likely have biological and medicinal significance. There are numerous oxidative stress-induced pathophysiologic conditions during which redox status and, in particular, the GSH/GSSG ratio is perturbed (43, 45–47). An important role for glutathione has been proposed for the pathogenesis of PD, where a decrease in GSH concentrations in the substantia nigra was observed in preclinical stages of the disease (48). Furthermore, mitochondrial dysfunction appears to play a major role in the neurodegeneration associated with the pathology of PD (25). We observed the presence of the GSS-protein adduct of IDPm purified by immunoprecipitation in brain samples from the PD mouse model. The possibility that regulation of IDPm by glutathionylation in many diseases related to oxidative stress is worthy of further consideration.

In conclusion, under conditions favoring protein glutathionylation such as oxidative stress, the inactivation of key antioxidant enzymes would further deteriorate cell homeostasis. Therefore, glutathionylation of IDPm could be considered an adaptation of the cell to severe oxidative stress.
Glutathionylation of IDPm

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