Mitochondrial Complex II in the Post-ischemic Heart

OXIDATIVE INJURY AND THE ROLE OF PROTEIN S-GLUTATHIONYLATION

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Mitochondrial superoxide (O$_2^-$) is an important mediator of ischemia/reperfusion (I/R) injury. The O$_2^-$ generated in mitochondria also acts as a redox signal triggering cellular apoptosis. The enzyme succinate ubiquinone reductase (SQR or complex II) is one of the major mitochondrial components hosting regulatory thiols. Here the intrinsic protein S-glutathionylation (PrSSG) at the 70-kDa FAD-binding subunit of SQR was detected in rat heart and in isolated SQR using an anti-GSH monoclonal antibody. When rats were subjected to 30 min of coronary ligation followed by 24 h of reperfusion, the electron transfer activity (ETA) of SQR in post-ischemic myocardium was significantly decreased by 41.5 ± 2.9%. The PrSSGs of SQR-70 kDa were partially or completely eliminated in post-ischemic myocardium obtained from in vivo regional I/R hearts or isolated global I/R hearts, respectively. These results were further confirmed by using isolated succinate cytochrome c reductase (complex II + complex III). In the presence of succinate, O$_2^-$ was generated and oxidized the SQR portion of SCR, leading to a 60–70% decrease in its ETA. The gel band of the S-glutathionylated SQR 70-kDa polypeptide was cut out and digested with trypsin, and the digests were subjected to liquid chromatography/tandem mass spectrometry analysis. One cysteine residue, Cys$^{90}$, was involved in S-glutathionylation. These results indicate that the glutathione-binding domain, $^{77}$AAFGLEAGFNTACVTK$^{93}$ (where underline indicates Cys$^{90}$), is susceptible to redox change induced by oxidative stress. Furthermore, in vitro S-glutathionylation of purified SQR resulted in enhanced SQR-derived electron transfer efficiency and decreased formation of the 70-kDa-derived protein thyl radical induced by O$_2^-$ Thus, the decreasing S-glutathionylation and ETA in mitochondrial complex II are marked during myocardial ischemia/reperfusion. This redox-triggered impairment of complex II occurs in the post-ischemic heart and should be useful to identify disease pathogenesis related to reactive oxygen species-induced mitochondrial dysfunction.

Mitochondrial dysfunction in ischemia-reperfusion injury is caused by oxidative stress (1–6). In the post-ischemic heart, oxygen delivery to the myocardium is not sufficient to meet the need for mitochondrial oxidation during the physiological conditions of hypoxia, leaving the mitochondrial electron transport chain (ETC) in a more reduced state. This results in increased electron leakage from the ETC that in turn reacts with residual molecular oxygen to give O$_2^-$(7, 8). Because of the lack of ADP, re-introduction of oxygen with reperfusion will greatly increase electron leakage along with a decrease in scavenging capacity, leading to O$_2^-$ and O$_2^-$-derived oxidants being overproduced in mitochondria. Specifically, an increased hyperoxygenation induced by reperfusion in the post-ischemic heart has been detected by in vivo EPR oximetry (9). The overproduction of ROS also initiates oxidative inactivation of the ETC as reported previously (9).

Mitochondrial complex II (succinate:ubiquinone oxidoreductase; EC 1.3.5.1) is a key membrane complex in the tricarboxylic acid cycle that catalyzes the oxidation of succinate to fumarate in the mitochondrial matrix (10). Succinate oxidation is coupled to reduction of ubiquinone at the mitochondrial inner membrane as one part of the respiratory electron transport chain. SQR mediates electron transfer from succinate to ubiquinone through the prosthetic groups of FAD as follows: [2Fe-2S]$^0$(S1), [4Fe-4S]$^0$(S2), and [3Fe-4S]$^0$(S3) and heme b. The enzyme is composed of two parts as follows: a soluble succinate dehydrogenase and a membrane-anchoring protein fraction. Succinate dehydrogenase contains two protein subunits, a 70-kDa protein with a covalently bound FAD, and a 30-kDa iron-sulfur protein hosting S1, S2, and S3 iron-sulfur clusters (10). The membrane-anchoring protein fraction contains two hydrophobic polypeptides (14 and 9 kDa) with heme b binding (11).

The catalysis of SQR is believed to contribute to superoxide generation in mitochondria (12–16). Two regions of the

2 The abbreviations used are: ETC, electron transport chain; I/R, ischemia/reperfusion injury; SQR, succinate ubiquinone reductase or mitochondrial complex II; SCR, succinate cytochrome c reductase or supercomplex containing complex II and complex III; CQR, ubiquinol cytochrome c reductase or complex III; GS-SQR, glutathionylated SQR; O$_2^-$, superoxide anion; ROS, SMP, sub mitochondrial particles; DEPMPO, 5-dietoxylphosphoryl-5-methyl-1-pyrrrole N-oxide; DMO, 5,5-dimethyl pyrrrole N-oxide; SOD, superoxide dismutase; LC, liquid chromatography; MS/MS, tandem mass spectrometry; PBS, phosphate-buffered saline; ETA, electron transfer activity; DCPPI, dichlorophenol indophenol; p-ME, β-mercaptoethanol; DTT, dithiothreitol; LAD, left atrial descending; PrSSG, protein S-glutathionylation; DTPA, diethylentriaminepentaacetic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Q$_o$, ubiquinone-2; TTC, 2,3,5-Trienyltetrazolium chloride; GS, glutathione; ROS, reactive oxygen species.

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enzyem complex are hypothesized to be responsible for generating the superoxide anion radical, O$_2^-$. One is located on the FAD cofactor and is modulated by FADH$_2$ semiquinone (15, 17), whereas the other is likely located on the ubiquinone-binding site, which acts in the mediation of ubiquinone reduction (13, 14). Defects in the SQR leading to O$_2^-$ overproduction have been linked with the pathogenesis of certain diseases.

The redox pool in mitochondria is enriched in GSH with a physiological concentration of 5–10 mM in liver mitochondria (18). However, lower GSH concentrations of 0.5–2 mM are present in other tissues, including heart, and generally mitochondrial GSH concentrations are similar to those in the cytosol (19). The 70-kDa subunit of SQR is one of the major components to host reactive/regulatory thiols, which are thought to have biological functions of antioxidant defense and redox signaling. The physiological role of SQR-derived regulatory thiols at the 70-kDa subunit has been implicated in the regulation of respiration and nitric oxide utilization (20).

An important response of protein thiols (PrSH) to oxidative stress is to reversibly form protein mixed disulfides (PrSSG) via S-glutathionylation (21–25). This post-translational modification has been suggested as a common mechanism regulating protein functions related to physiological changes (22, 26). However, the molecular mechanism of protein S-glutathionylation of SQR and how this redox modification affects the enzymatic functions of SQR remain unknown. Myocardial ischemia/reperfusion provides an outstanding disease model to probe mitochondrial dysfunction resulting from in vivo overproduction of oxygen-free radical(s).

This study was undertaken to address the fundamental questions regarding the redox biochemistry of SQR and its molecular mechanism of damage in the post-ischemic heart. Here we focused on the 70-kDa flavin subunit of SQR. We observed marked loss of SQR activity in the post-ischemic myocardium. This decreased activity was associated with deglutathionylation of the 70-kDa FAD-binding subunit of SQR, suggesting S-glutathionylation activates the enzyme in vivo. To further understand the mechanism of SQR glutathionylation, in vitro studies were performed to identify the cysteine residue involved in the protein S-glutathionylation of SQR and functionally characterize glutathionylated SQR, including its electron transport and superoxide generation activities that result from protein S-glutathionylation.

**MATERIALS AND METHODS**

**Reagents**

Ammonium sulfate, diethylenetriaminepentaacetic acid (DTPA), ubiquinone-2 (Q$_2$), sodium cholate, deoxycholic acid, glutathione (GSH), oxidized glutathione (GSSG), Triton X-100, carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone (FCCP), oligomycin, Cu,Zn-SOD and sodium succinate were purchased from Sigma and used as received. The 5-diethylphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO) spin trap and anti-DMPO polyclonal antibody were purchased from Alexis Biochemicals (San Diego, CA). The DMPO spin trap from Aldrich was vacuum-distilled twice and stored under nitrogen at −80 °C until needed.

**Isolated Rat Hearts Model**

Global ischemia-reperfusion conditions were imposed upon isolated hearts according to published procedures (27, 28). Briefly, male Sprague-Dawley rats (~300–350 g and 9–10 weeks old) were heparinized with heparin (500 units) and anesthetized with intraperitoneal Nembutal (30–35 mg/kg). The hearts were excised, and the aortas were cannulated and perfused at 37 °C with a constant pressure of 80 mm Hg using Krebs solution (in mM, 16.7 glucose, 120 NaCl, 25.0 NaHCO$_3$, 2.5 CaCl$_2$, 5.9 KCl, 0.5 EDTA, and 1.2 MgCl$_2$) pre-bubbled with 95% CO$_2$, 5% O$_2$ gas. Two side arms in the perfusion line were located just proximal to the heart cannula can allow infusion of treatment agents. Hearts were subjected to control perfusion or 30 min of ischemia followed by 60 min of reperfusion. Hearts were then placed on ice and immediately subjected to mitochondrial preparation. Ischemic duration was 30 min. The isolated hearts were perfused for 60 min at 37 °C by the method of Langendorf at a constant pressure of 80 mm Hg with a Krebs-buffered perfusate.

**In Vivo Myocardial I/R Model**

The procedure for the in vivo ischemia-reperfusion rat model was performed by the technique reported in the literature (9, 29) as indicated in supplemental Fig. 1. Sprague-Dawley rats (~300–350 g and 9–10 weeks old) were anesthetized with intraperitoneal Nembutal (80–100 mg/kg). After the rats were fully anesthetized, they were intubated and then ventilated with air (1.0 ml, rate of 100 breaths/min) using a mechanical ventilator model 683 (Harvard Apparatus, Holliston, MA). The rats then underwent a left lateral thoracotomy; the pericardium was opened, and a pericardial cradle was formed to allow adequate exposure of the heart surface. The left anterior descending (LAD) coronary artery was then occluded by placing a suture (6.0 nylon) around the origin of the LAD.

After 30 min of ischemia, the suture around the coronary artery was untied, allowing reperfusion to occur. Following the reperfusion, all wounds were closed and infiltrated with 0.5% xylocaine (~0.3 ml). The muscular layers and skin incisions were closed with 4.0 nylon sutures. A chest tube (2.5-cm polyethylene 50 tubing) was inserted at the wound site and maintained in position while the animal was taken off respiratory support.

Upon spontaneous breathing, the chest tube was removed and a surgical clip applied over the withdrawal site. The animal was allowed to recover, and a physiological assessment was performed. During the recovery period the animals received supportive post-operative care as needed. Body temperature was maintained at 37 °C by a thermal heating pad. By h post-operation, the animals had recovered sufficiently to eat and drink independently.

At 1 day post-infarction, the rats were placed under deep anesthesia with Nembutal (200 mg/ml). The rats were then sacrificed, and the hearts excised and placed in the PBS buffer. The infarct area was identified by 2,3,5-triphenyltetrazolium chloride (TTC) staining. The risk region of myocardial tissue without TTC staining was excited and subjected to biochemical analysis.
Preparation of Mitochondrial Succinate Cytochrome c Reductase (SCR) and Succinate Ubiquinone Reductase (SQR)

Bovine heart mitochondrial SCR was prepared from submitochondrial particles (SMP). SMP was suspended in 0.1 M borate/phosphate, pH 7.8, to cytochrome b content of 11 \( \mu \text{M} \) and solubilized with sodium cholate (4 ml of 20% sodium cholate, 100 ml of SMP suspension).

Solid ammonium sulfate was added to 37.5% saturation (21.25 g/100 ml) and stirred for 60 min before being centrifuged at 21,600 \( \times g \) for 90 min in a Beckman J30-I centrifuge, rotor JLA 16.25. The supernatant was collected to bring to 50% ammonium sulfate saturation (8.9 g/100 ml) and centrifuged at 21,600 \( \times g \) in the same centrifuge for 20 min. The pellet collected was dissolved in 50 mM sodium/potassium phosphate buffer, pH 7.4, containing 0.67 M sucrose (TS buffer). Further purification of SCR was achieved by repeat ammonium acetate fractionation in the presence of deoxycholate. The crude SCR was diluted in TS buffer to a protein concentration of \( \sim 20 \) mg/ml (estimated by 0.75 absorption at 278 nm in 1% SDS), and the protein was solubilized with deoxycholate (0.35 mg/mg protein). A 50% saturated solution of ammonium acetate was used. The purified SCR was collected and dialyzed against 50 mM Tris-Cl, pH 7.2, containing 0.2% sodium cholate and 10% glycerol. The specific activity of purified SCR is \( \sim 9.1 \) \( \mu \text{mol of cytochrome c} \) reduced per min/mg protein.

SQR was isolated from succinate cytochrome c reductase (SCR) by calcium phosphate-cellulose chromatography under nonreducing conditions according to the published method developed by Yu and co-workers (30, 31). Succinate was omitted from all buffers used in the purification procedure. SQR-containing fractions obtained from the second calcium phosphate-cellulose column were concentrated by 43% ammonium sulfate saturation and centrifuged at 48,000 \( \times g \) for 20 min (31). The precipitate obtained was dissolved in 50 mM sodium/potassium phosphate, pH 7.8, containing 0.2% sodium cholate and 10% glycerol. The specific activity of purified SQR is \( \sim 15.2 \) \( \mu \text{mol of succinate oxidized (or DCPIP reduction)/min/mg protein} \).

Analytical Methods

Optical spectra were measured on a Shimadzu 2401 UV-visible recording spectrophotometer. The protein concentrations of SMP and rat heart tissue homogenates were determined by the Lowry method using bovine serum albumin as a standard. The heme b concentration of SQR was calculated from the differential spectrum between dithionite reduction and ferricyanide oxidation. The concentration of Q2 was determined by absorbance spectra from NaBH4 reduction using a millimolar extinction coefficient \( \epsilon_{275-290\text{nm}} = 12.25 \text{M}^{-1}\text{cm}^{-1} \) (32). The enzyme activity of SQR was assayed by measuring Q2-stimulated DCPIP reduction by succinate as described in the literature (31).

To measure the electron transfer activity of SQR, an appropriate amount of SQR was added to an assay mixture (1 ml) containing 50 mM phosphate buffer, pH 7.4, 0.1 mM EDTA, 75 \( \mu \text{M} \) DCPIP, 50 \( \mu \text{M} \) Q2, and 20 \( \mu \text{M} \) succinate as described by Hatefi et al. (33). The SQR activity was determined by measuring the decrease in absorbance at 600 nm. The specific activity of SQR (nmol of DCPIP reduction (or succinate oxidized)/min/mg of SQR) was calculated using a molar extinction coefficient \( \epsilon_{560\text{nm}} = 21 \text{M}^{-1}\text{cm}^{-1} \). QCR (complex III) activity was assayed as reported previously (34–36). An appropriate amount of SCR or tissue homogenate was added to an assay mixture (1 ml) containing 50 mM sodium/potassium phosphate buffer, pH 7.0, 1 mM EDTA, 50 \( \mu \text{M} \) cytochrome c, and 25 \( \mu \text{M} \) Q2. QCR activity was determined by monitoring the reduction of cytochrome c (the increase in absorbance at 550 nm) at room temperature.

Mitochondria Preparation and Oxygen Consumption Measurements

Mitochondria were prepared by differential centrifugation described in the published method (37) with modifications. Mitochondria were precipitated by centrifugation at 20,000 \( \times g \) for 10 min at the final step and resuspended in the media containing 230 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 5 mM Trizma (Tris base)/HCl buffer, pH 7.4 (M-buffer). Mitochondrial respiration was measured by the polarographic method using a Clark-type oxygen electrode (World Precision Institute, Sarasota, FL). The incubation media contained M-buffer at 37 or 30 °C. Mitochondrial preparations were added to the media at a concentration of 0.25 mg/ml, and oxygen consumption was induced by succinate (5 mM). ADP (0.5 mM) was then added, and the following parameters were measured as follows: state 2, oxygen consumption before the addition of ADP; state 3, oxygen consumption stimulated by ADP; state 4, oxygen consumption after the addition of oligomycin (6 \( \mu \text{g}/\text{mg mitochondria}) followed by ADP addition. The oxygen electrode was calibrated by assuming the concentration of O2 in the incubation medium at 37 °C to be 200 \( \mu \text{M} \) or 30 °C to be 250 \( \mu \text{M} \).

Immunoblotting Analysis

Myocardial tissues excised from the normal region (or nonischemic area) and risk region (or infarction area) containing the LAD were minced and homogenized with a Polytron homogenizer (250 watts, 10 s, three times) in ice-cold HEPES buffer (3 mM, pH 7.2) containing sucrose (0.25 M), EGTA (0.5 mM), and protease/inhibitor mixture (1:40; Roche Diagnostics). The supernatant of tissue homogenate was collected by centrifugation at 600 \( \times g \) for 20 min. The reaction
mixture was mixed with the Laemmli sample buffer at a ratio of 4:1 (v/v) in the presence of β-ME (for detection of 70-kDa and DMPO adduct) or in the absence of β-ME (for detection of S-glutathionylation), incubated at 70 °C for 10 min, and then immediately loaded onto a 4–20% Tris-glycine polyacrylamide gradient gel. Samples were run at room temperature for 2 h at 100 V. Protein bands were electrophoretically transferred to nitrocellulose membranes in 25 mM Tris, 192 mM glycine, and 10% methanol. Membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 (TTBS) and 5% dry milk (Bio-Rad). The blots were then incubated overnight with anti-GSH monoclonal antibody (ViroGen Corp., Watertown, MA), anti-70-kDa monoclonal antibody (Molecular Probes, Inc. Eugene, OR), or anti-DMPO polyclonal antibody at 4 °C. Blots were then washed three times in TTBS and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG in TTBS at room temperature. The blots were again washed twice in TTBS and twice in Tris-buffered saline, and then visualized using ECL Western blotting detection reagents (Amersham Biosciences).

Mass Spectrometry
The sample of S-glutathionylated SQR (GS-SQR) was prepared by incubated with as-isolated SQR (2 μM based on heme b) with GSSG (1 mM) at room temperature for 1 h. To avoid thiol-disulfide exchange and prevent artifactual oxidation, the free thiols of GS-SQR was subjected to carbomylmethylation with iodoacetamide (2 mM) at room temperature for several hours, the gel bands were dehydrated with acetonitrile and washed again with cycles of acetonitrile and 100 mM hydrochloric acid for 30 min for rehydration before the addition of another 20 μl aliquot of trypsin (20 ng/μl) or chymotrypsin (25 ng/μl) in 50 mM ammonium bicarbonate buffer. Protein bands on the gel were then stained for 1 h with Coomassie Blue, and the background of staining was rapidly removed by methanol/acetic acid/water (40:10:50). The gel was then equilibrated with distilled water at 4 °C overnight prior to in-gel digestion and MS measurement.

In-gel Digestion—Gels were digested with sequencing grade trypsin (Promega, Madison WI) and chymotrypsin (Roche Diagnostics) using the Montage in-gel digestion kit from Millipore (Bedford, MA) following the manufacturer’s recommended protocols with minor changes for optimization of peptide extraction. Briefly, the bands of interest were trimmed as closely as possible to minimize background polyacrylamide gel. After being washed twice in 50% methanol, 5% acetic acid for several hours, the gel bands were dehydrated with acetonitrile and washed again with cycles of acetonitrile and 100 mM ammonium bicarbonate buffer. The gels were then dried of 4–20% gradient Tris-glycine polyacrylamide gel. Protein bands on the gel were then stained for 1 h with Coomassie Blue, and the background of staining was rapidly removed by methanol/acetic acid/water (40:10:50). The gel was then equilibrated with distilled water at 4 °C overnight prior to in-gel digestion and MS measurement.

Nano-LC/MS/MS (LC/MS/MS)—Capillary-liquid chromatography tandem mass spectrometry (nano-LC/MS/MS) was performed on a Micromass hybrid quadrupole time-of-flight Q-Tof II (Micromass, Wythenshawe, UK) mass spectrometer equipped with an orthogonal nanospray source (New Objective, Woburn, MA) operated in positive ion mode. The capillary LC system was a Dionex UltiMate system (Dionex, Sunnyvale, CA). Solvent A was water containing 50 mM acetic acid, and solvent B was acetonitrile. A 5-cm 75 μm ID BioBasic C18 column (New Objective, Woburn, MA) packed directly in the nanospray tip was used for chromatographic separations. 2.5-μl aliquots of each sample were injected onto the column for the analysis.

Peptides were eluted directly off the column into the quadrupole-time-of-flight system using a gradient of 2–80% solvent B over 48 min, with a flow rate of ~300 nl/min. The total run time was 55 min. The nanospray capillary voltage was set at 3.0 kV and the cone voltage at 40 V. The source temperature was maintained at 100 °C.

Mass spectra were acquired from m/z 400–2,000 every 0.9 s with a resolution of 8,000 (full width at half-maximum) and recorded using MassLynx 4.0 with automatic switching functions. When the desired peak was detected at a minimum of 15 ion counts, the mass spectrometer automatically switched to acquire a collision-induced dissociation spectrum of the individual peptide; mass spectra were acquired from m/z 75–2000 to detect immonium ions. Collision energy was set depending on charge state recognition properties.

Sequence information from the MS/MS data were processed using the Mascot Distiller software with standard data processing parameters. Data base searches were performed using the Mascot (Matrix Science, Boston) and PEAKS programs (Bioinformatics Solutions, Waterloo, Ontario, Canada).

Electron Paramagnetic Resonance Measurements
EPR measurements were performed on a Bruker EMX spectrometer operating at 9.86 GHz with 100 kHz of modulation frequency at room temperature. The reaction mixture was transferred to a 50-μl capillary, which was then positioned in the high sensitivity cavity (Bruker Instrument, Billerica, MA). The sample was scanned using the following parameters: center field, 3510 G; sweep width, 140 G; power, 20 milliwatts; receiver gain, 2 × 105; modulation amplitude, 1 G; time of conversion, 163.84 ms; time constant, 163.84 ms; number of scans, three. The spectral simulations were performed using the WinSim program developed at NIEHS, National Institutes of Health, by Duling (38). The hyperfine coupling constants used to simulate the spin adduct of DEPMPO/OOHH were as follows: isomer 1, aN = 13.14 G, aH = 11.04 G, aHH = 0.96 G, aH = 49.96 G (80% relative concentration); isomer 2, aN = 13.18 G, aH = 12.59 G, aHH = 3.46 G, aH = 48.2 G (20% relative concentration) (32, 39).

RESULTS
State 3, State 4, and Mitochondrial Integrity in the Post-ischemic Rat Heart—It has been suggested that overproduction of oxygen free radicals during ischemia/reperfusion injury increases Ca2+ accumulation, leading to depolarization of the membrane and uncoupling of respiration (40–42). Therefore, we measured the respiratory control ratio of mitochondrial...
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**FIGURE 1. State 3 and state 4 respiratory rates of mitochondrial preparations from normal and post-ischemic rat hearts.** Post-ischemic heart was obtained through the conditions of global ischemia (30 min)-reperfusion (60 min) injury as described under “Materials and Methods.” The oxygen consumption by mitochondria was measured by oxygen polarograph at 37 °C as described under “Materials and Methods.” Mitochondria; Suc., succinate.

**FIGURE 2. State 3 and State 4 respiratory rates of tissue homogenates prepared from normal and post-ischemic myocardium.** Post-ischemic myocardium was obtained through the conditions of in vivo regional ischemia (30 min) and reperfusion (24 h) as described under “Materials and Methods” (supplemental Fig. 1). The tissue of risk region was excised, minced, and homogenized in the M-buffer containing nargase (1 mg/g tissue). Homogenates were subjected to centrifugation at 800 g for 8 min. The supernatant (0.5 mg/ml) was treated with phenylmethylsulfonyl fluoride (1 mM) prior to oxygen consumption measurement as described under “Materials and Methods.” Suc., succinate.

Preparation from the post-ischemic heart. The procedure of global ischemia (30 min)/reperfusion (60 min) rat heart model was performed as described under “Materials and Methods.” The hearts were subjected to mitochondrial preparation.

Fig. 1A shows a typical profile of oxygen consumption in mitochondria from the normal and the global ischemic heart. As indicated in Fig. 1B, ADP-stimulated respiration (state 3) was decreased from 368.8 to 186.9 nmol of O2/min/mg of protein after global ischemia/reperfusion injury. The respiratory control ratio (defined as the ratio of state 3 to state 4) was decreased from 4.4 to 2.8, indicating defects in the mitochondrial integrity of the post-ischemic heart.

To access the maximal electron transfer activity of isolated mitochondria, FCCP (2 μM) was added to uncouple the mitochondria and maximize the respiratory capacity, and oxygen consumption was initiated by succinate (5 mM) in the absence of ADP. As shown in Fig. 1B, FADH2-dependent electron transfer activity was decreased from 277.9 to 88.2 nmol of O2/min/mg of protein, indicating oxidative injury to the electron transport chain during the global ischemia/reperfusion injury.

Oxidative Injury of Mitochondrial SQR in the Post-ischemic Rat Heart—To provide further evidence for the oxidative impairment of FADH2-dependent electron transfer activity (Fig. 1B), further studies were performed in an in vivo model of regional ischemia and reperfusion. Rats were subjected to 30 min of coronary ligation followed by 24 h of reperfusion (supplemental Fig. 1A). Myocardial infarction was examined by TTC staining (supplemental Fig. 1B). Myocardial tissue (without TTC staining) was excised from the risk region (or infarction area) containing the LAD and homogenized in ice-cold HEPES buffer (3 mM, pH 7.2) containing sucrose (0.25 M), EGTA (0.5 mM), and protease/inhibitor mixture (1:40, Roche Diagnostics). The tissue homogenate was immediately subjected to analysis of respiration driven by succinate at 30 °C as described under “Materials and Methods.” The respiratory control ratio was decreased from an average of 3.4 (for normal tissue) to 1.1 (tissue with myocardial infarction), suggesting a nearly complete loss of ADP-stimulated respiration in the risk region of post-ischemic heart (Fig. 2). In the presence of FCCP (2 μM), FADH2-dependent oxygen consumption was decreased ~49% from 155.2 to 61.9 nmol of O2/min/mg of protein, thus confirming impairment of electron transfer from succinate to molecular oxygen because of myocardial infarction (Fig. 2).

The enzymatic activities of succinate cytochrome c reductase (SCR, complex II + complex III), SQR (complex II), and QCR (complex III) were measured at room temperature as described under “Materials and Methods.” The specific activities of SCR, SQR, and QCR in the tissue homogenate of normal heart were 82.6 nmol of ferricytochrome c reduction/min/mg protein, 68.0 nmol of DCPIP reduction/min/mg protein, and QCR, 153.4 nmol of ferricytochrome c reduction/min/mg protein.

As indicated in Fig. 3, significant impairment of electron transfer activity was detected in SCR and SQR. However, oxidative inactivation of QCR was not detected in the tissue homogenate of the post-ischemic heart, suggesting that the oxidative injury to SCR was because of the impairment of SQR. The electron transfer activities of SQR and SCR detected in the post-ischemic heart were sensitive to threonyl trifluoroacetone (1 mM), and those of SCR and QCR were sensitive to the antimycin A (10 μM) treatment.
It should be noted that the QCR activity in tissue homogenate was enhanced by 20–35% when the sodium cholate (0.05%) was included in the assay mixture. However, structural/functional damage on ischemia-reperfusion to the QCR activity in the tissue homogenate is not significant when the assay of enzymatic activity was carried out in the presence or absence of detergent.

**Superoxide Production by SCR-induced Self-inactivation with the Impairment of SQR**—The observation of oxidative injury of SQR and SCR in the post-ischemic heart was further confirmed in the system of isolated SCR (Fig. 4). Incubation of SCR (2 µM) with succinate (18 µM) at room temperature resulted in superoxide generation as detected by EPR (12) and progressive loss of ETA of SCR and SQR (Fig. 4, A and B). However, the ETA of QCR did not suffer progressive loss (Fig. 4C), indicating that the impairment of SQR is accounted for by the self-inactivation of SCR. The addition of SOD partially protected SCR from oxidative damage (Fig. 4D, dotted curve and △), suggesting the involvement of superoxide anion radical (O$_2^-$). However, the oxidative damage was completely prevented by a combination of SOD and catalase (Fig. 4D, dashed curve and ●), suggesting the involvement of H$_2$O$_2$ presumably derived from the spontaneous dismutation of superoxide.

**Protein S-Glutathionylation of the 70-kDa Flavin Subunit of SQR in the Post-ischemic Myocardium**—Tissue homogenates (100 µg) were directly probed with a monoclonal antibody against 70-kDa FAD-binding protein of SQR, and glyceraldehyde-3-phosphate dehydrogenase was used as a loading control for Western blotting. There was no significant change observed in the protein expression level of the 70-kDa protein from the post-ischemic myocardium (data not shown).

It has been proposed that SQR is one of the major components in mitochondria to host protein thiols; the 70-kDa flavin subunit is known to contain reactive/regulatory thiol(s) and implicate the SQR as a host of the redox thiols (20, 43). Tissue homogenates (300 µg) were subjected to immunoprecipitation with anti-70-kDa monoclonal antibody, and subsequently subjected to SDS-PAGE and immunoblotted with anti-70-kDa and anti-GSH monoclonal antibodies. As indicated by Western blots (under nonreducing conditions) (Fig. 5A), we have detected the existence of intrinsic protein S-glutathionylation on the 70-kDa subunit of SQR from the tissue homogenate of normal hearts using anti-GSH antibody. The detected SQR-derived PrSSG at 70-kDa was subsequently decreased.
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**A** *In vivo regional myocardial I/R (30-min I / 24-h R)*

![Image](Image 49x476 to 299x733)

**B** *Isolated heart-global I/R (30-min I / 1-h R)*

![Image](Image 342x609 to 423x647)

**Figure 5.** Protein S-glutathionylation of the 70-kDa subunit of SQR in the myocardial tissue homogenates and the effect of the post-ischemic injury. Myocardial tissue homogenates (300 μg) were subjected to immunoprecipitation with anti-70-kDa antibody, and subsequently subjected to SDS-PAGE and immunoblotted with anti-70-kDa (left-side panel) and anti-GSH antibodies (right-side panel). A, lane 1, protein from normal rat heart (NH). Lane 2, protein from post-ischemic rat heart (PH). Lane 3, purified SQR (40 pmol) as standard control. B, same as A except that post-ischemic heart was obtained through the conditions of regional ischemia (30 min)-reperfusion injury (60 min). Density ratio of the blotting signals between anti-GSH and anti-70 kDa was enhanced in proportion to the dosage of GSH (data not shown).

(~40%) in the post-ischemic myocardium obtained from the in vivo model of regional ischemia and reperfusion (Fig. 5A, right-side panel) and completely eliminated in the isolated heart subjected to 30-min global ischemia and 60-min reperfusion (Fig. 5B, right-side panel).

**Protein S-Glutathionylation of the 70-kDa Flavin Subunit of SQR Isolated from the Bovine Heart**—The above observation in the post-ischemic heart was further confirmed in a system with isolated SQR. Bovine SQR was isolated from SCR by calcium phosphate-cellulose chromatography under nonreducing conditions (30). We detected the presence of a small and variable amount of intrinsic protein GSH mixed disulfide (PrSSG) in isolated SQR at the 70-kDa subunit using an anti-GSH monoclonal antibody (Fig. 6A, lane 1). This result was consistent and reproducible in different batches of SQR preparations from the bovine heart (*n* = 5). The Western blot signal of intrinsic PrSSG at the 70-kDa subunit was subsequently diminished in the presence of β-ME (Fig. 6A, lane 2). The protein S-glutathionylation of the 70-kDa subunit was greatly enhanced by GSSG (1 mM) (Fig. 6A, lane 3) and diminished in the presence of β-ME (Fig. 6A, lane 4). The same preparation was further immunoblotted with monoclonal antibody against the 70-kDa subunit to ensure the same amount of protein loading as indicated in Fig. 6B. It should be noted that the intensity of the Western blot signal at the 70-kDa subunit was enhanced in proportion to the dosage of GSSG (data not shown).

**Figure 6.** Protein S-glutathionylation of the 70-kDa subunit of isolated SQR. SQR was isolated from the bovine heart according to the published procedure. Proteins (50 pmol, based on heme b) were subjected to SDS-PAGE and followed by immunoblotting with anti-GSH (A) and anti-70-kDa antibodies (B). A, lane 1, isolated native SQR. Lane 2, isolated native SQR pretreated with β-ME (1%) prior to SDS-PAGE. Lane 3, GS-SQR; purified SQR (2 μM) was incubated with GSSG (1 mM) at room temperature for 1 h. Lane 4, GS-SQR pretreated with β-ME (1%) prior to SDS-PAGE. B, same as A except that the proteins were probed with anti-70-kDa antibody. C, SQR was treated with DTT (1 mM) and the excess DTT was removed by a Sephadex G-25 column. The yielded DTT-SQR (1 μM) was subsequently incubated with various concentrations of GSH/GSSG mixture at 37 °C for 1 h. Protein (30 pmol) was subjected to SDS-PAGE and followed by immunoblotting with anti-GSH. D, same as C except that incubation was carried out under the conditions of room temperature and 24 h.

**Protein S-Glutathionylation of the Isolated SQR in the Presence of GSH**—To see whether GSSG-stimulated SQR-derived S-glutathionylation can take place in the presence of GSH, which is a similar condition of the mitochondrial milieu, isolated SQR was treated with dithiothreitol (1 mM) to remove intrinsic S-glutathionylation and then passed through a Sephadex G-25 column. The SQR (1 μM, based on heme b) was incubated in the reaction mixture containing different ratios of GSH/GSSG (GSH plus GSSG = 2 mM) at 37 °C for 1 h. As indicated in the Fig. 6C, the detection of 70-kDa-derived S-glutathionylation was significant when the ratio of GSSG/GSH was equal or higher than 0.6 (GSSG = 0.75 mM, GSH = 1.25 mM). The Western blot signal was increased in proportion to the amount of GSSG and decreased in proportion to GSH. However, the detection of S-glutathionylation at 70 kDa was gradually significant at a lower ratio of GSSG/GSH when the time of incubation was prolonged to 24 h at room temperature as shown in the Fig. 6D.

The protein band of the 70-kDa subunit of glutathionylated SQR (Fig. 6C, lane 5) was subjected to in-gel digestion with trypsin/chemotrypsin/Glu C and analyzed by LC/MS/MS. With this technique, 64.9% of the amino acid sequence of the 70-kDa subunit was identified in the MS/MS spectra of the tryptic/chemotryptic/Glu C peptides of SQR-70 kDa (data not shown). 5 of 18 cysteine residues were identified by LC/MS/MS under the nonreducing conditions in the absence of β-ME. However, 15 of 18 cysteine residues were identified by LC/MS/MS with 88.1% sequence coverage under the reducing conditions in the presence of β-ME. These results together indicated that the possible existence of disulfide bond linkage limited complete sequence coverage during LC/MS/MS analysis. Furthermore, we have detected three fragments involved in missing cleavage under nonreducing conditions (data not shown), which could also contribute to the limitation of...
sequence coverage by LC/MS/MS analysis because of the large peptide containing higher molecular weight.

The addition of one glutathione to native protein will increase the molecular weight of the protein by 305 Da. Therefore, the mass spectra from the proteolytic digest of PrSSG of the SQR-70-kDa polypeptide were examined for the addition of 305 Da to the tryptic or chymotryptic peptides. This mass difference was observed in the tryptic digest MS/MS results, indicating that the mass shift of 305 Da occurred on the specific amino acid residue, Cys90. The tryptic peptide was identified as the fragment 77AAFGLSEAGFNTACVTK93 (where underline indicates Cys90), named GSC90 (Fig. 7, and Table 1).

The doubly protonated molecular ion (M + 2H)+ of tryptic peptide GSC90 was observed at m/z 996.63, which has a mass shift of 305 Da compared with the parent ion (m/z 1686.8161 for (M + H)+). These data suggest that one GSH is covalently bound to one of the residues of the GSC90 peptide.

To determine which amino acid(s) were covalently linked to the GSH in the GSC367 peptide, the MS/MS spectrum of the (GSC90)2+ ion at m/z 996.63 was obtained. As shown in Fig. 7 and Table 1, under the conditions of low energy collision-induced dissociation, both y and b product ions were observed, corresponding to cleavages along the peptide backbone (44, 45). The y series ions result from C-terminal peptide cleavages, whereas the b series ions result from cleavages at the N terminus.

In the spectrum of the (GSC90)2+ ion (Fig. 7), some of the structurally informative fragment ions, including y4–y5, y7–y14, and b14–b16, were observed with a mass shift of 305 Da compared with the native fragment ions, thus allowing unequivocal assignment of the glutationylated adduct to the cysteine 90 residue of the tryptic peptide 77AAFGLSEAGFNTACVTK93 (underline indicates Cys90). Other sequence informative ions including b4–b13 and y3 provided the evidence to ensure that the sequence of (GSC90)2+ was matched to the sequence of aa residues 77–93 of the 70-kDa subunit of the SQR.

It should be noted that LC/MS/MS analysis of GS-SQR prepared by incubation of SQR with 1.0 mM GSSG at room temperature for 1 h (Fig. 6A, lane 3) revealed a triply protonated molecular ion (GSC90)3+ at m/z 664.81, indicating the fragment 77AAFGLSEAGFNTACVTK93 is glutathionylated at Cys90 (underlined) (data not shown). Together the S-glutathionylation of Cys90 of the 70-kDa subunit can be confirmed in the presence or absence of GSH by LC/MS/MS. Therefore, Cys90 glutathionylation should be physiologically relevant.
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**TABLE 1**

| Δm between yn and yn-1 | Fragment ion | Measured m/z | Sequence | Measured m/z | Fragment ion | Δm between bn and bn-1 |
|------------------------|--------------|--------------|----------|--------------|--------------|-----------------------|
| 147.91                 | y15          | 925.72
| 57.09                  | y14          | 851.99                  |
| 112.97                 | y13          | 1645.44                  |
| 87.00                  | y12          | 1532.47                  |
| 129.04                 | y11          | 1445.47                  |
| 71.04                  | y10          | 1316.43                  |
| 57.04                  | y9           | 1245.39                  |
| 146.97                 | y8           | 1188.42                  |
| 214.98                 | y7           | 1041.40                  |
| 71.13                  | y5           | 826.42                   |
| 408.05 (103 + 305)     | y4           | 755.39                   |
|                       | y3           | 347.35                   |

**Superoxide Generation Mediated by SQR Detected by EPR Spin Trapping**—There is a growing body of evidence that links overproduction of O₂⁻ with a defect in SQR (13, 46–48). Reports from the literature have indicated that SQR plays a pathological role in a wide variety of diseases such as optic atrophy, tumor formation, myopathy, and encephalopathy (49, 50). To obtain direct evidence of such overproduction, mediation of O₂⁻ production by SQR was measured by EPR spin trapping with DEPMPO. When purified SQR (1 μM, based on heme b concentration) was incubated with DEPMPO (20 mM) in PBS and the reaction was initiated by the addition of succinate (18 μM), a multiline EPR spectrum was produced that was characteristic of DEPMPO/•OOH (Fig. 8A, *solid line*) based on hyperfine coupling constants obtained from computer simulation (Fig. 8A, *dashed line*). Trapping of SQR-mediated O₂⁻ by DEPMPO was thus highly specific and suitable for quantitative analysis.

In the absence of SQR, no DEPMPO/•OOH was detected (Fig. 8D), indicating the enzymatic dependence of the DEPMPO/•OOH adduct formation. When SQR was replaced with heat-denatured (70 °C, 5 min) SQR, the formation of DEPMPO/•OOH was inhibited (data not shown), indicating that electron transfer activity in the enzyme is required for O₂⁻ generation. The detection of the O₂⁻ adduct of DEPMPO was strongly inhibited when succinate was omitted from the system (Fig. 8C), suggesting a direct role for succinate as the electron donor for SQR-mediated O₂⁻ production.

The enzymatic activity of SQR is sensitive to TTFA, which inhibits electron transfer activity through binding to the S3 center and cytochrome b₅₆₀-binding protein. The addition of TTFA (1 mM) inhibited the electron transfer activity of SQR by more than 99% and inhibited O₂⁻ production up to 57% (n = 3, Fig. 8D), indicating that O₂⁻ generation can be derived from electron leakage from the reduced FAD moiety of the SQR 70-kDa subunit. The addition of ubiquinone-2 (Q₂) progressively diminished O₂⁻ production, and the maximal inhibition was observed to be 85% (Fig. 8E), indicating that 15% of the electron leakage could occur under enzyme turnover conditions when Q₂ was used as an electron acceptor.

**Effect of Protein S-Glutathionylation on the ETA and Superoxide Generation Activity of SQR**—Protein S-glutathionylation has been implicated as a mechanism to regulate the functions of the protein (26, 51, 52). Therefore, significant changes in the electron transfer activity and superoxide generation activity of SQR are expected because of the site-specific S-glutathionylation involved in the 70-kDa flavin protein subunit.

To learn the effect of this redox modification on the mediation of SQR-derived electron transfer activity and superoxide generation activity, we incubated SQR (1 μM based on heme b) in PBS with 1 mM GSSG at room temperature for 1 h and determined the electron transfer activity assayed for its ability to catalyze Q₂-stimulated DCPIP reduction by succinate. It was observed that specific S-glutathionylation of SQR marginally...
enhanced its electron transfer activity by $119 \pm 4\%$ ($n = 3$, and Fig. 9C).

SQR-mediated $O_2^-$ production under enzyme turnover conditions was analyzed by EPR spin trapping with DEPMPO. As indicated in Fig. 9, A and B, the $S$-glutathionylated SQR (GS-SQR) obtained was allowed to generate $O_2^-$ under conditions of enzyme turnover (in the presence of succinate and $Q_2$), and the products were analyzed by EPR spin trapping and subsequent spin quantitation based on the spectra obtained from computer simulation (Fig. 9, A and B, dashed line). The signal intensity from the DEPMPO/•OOH adduct mediated by GS-SQR was decreased to $68 \pm 6\%$ ($n = 3$) of that mediated by SQR. This result implies that protein $S$-glutathionylation enhanced the electron transfer efficiency and decreased the subsequent electron leakage for $O_2^-$ generation mediated by SQR.

The above results were further confirmed by the cytochrome $c$ reduction assay as indicated in Fig. 10. The purified SQR (5 $\mu$M, based on heme $b$) was incubated with GSSG (1 mM) at room temperature for 1 h. Cytochrome $c$ reduction was initiated by the addition of native SQR or GS-SQR (0.1 $\mu$M) to an assay mixture containing succinate (1 mM) and cytochrome $c$ (50 $\mu$M) in 50 mM phosphate buffer, pH 7.4. $O_2^-$-mediated cytochrome $c$ reduction was measured by pre-addition of Cu,Zn-SOD (300 units) in the same assay mixture. It was calculated that 3.0 nmol of cytochrome $c$ reduction/min/nmol of heme $b$ was mediated through $O_2^-$ generated by SQR, and 1.9 ($\sim 65\%$) nmol of cytochrome $c$ reduction/min/nmol of heme $b$ was mediated through $O_2^-$ generated by GS-SQR.

It should be noted that the ETA of GS-SQR was progressively enhanced in proportion to the dosage of GSSG used. In contrast, the superoxide generation activity of GS-SQR was gradually decreased in proportion to the dosage of GSSG used (data not shown). Presumably, this is because of increasing the extent of $S$-glutathionylation at the 70-kDa subunit of SQR.

Superoxide Production by SQR-induced Self-inactivation with Protein Radical Formation at the 70-kDa Subunit—$O_2^-$ production by SQR also induced self-inactivation as seen in the SCR (Fig. 4). A mechanism in which $O_2^-$ may induce oxidative attack on the protein matrix of SQR, forming the protein radical, is logically hypothesized. Immuno-spin trapping with anti-DMPO polyclonal antibody was used to define SQR-derived protein radical formation as described previously (32, 53, 54). Isolated SQR was treated with dithiothreitol (1 mM) to remove intrinsic $S$-glutathionylation and then passed through a Sephadex G-25 column. SQR (1 $\mu$M) was incubated with a nitro spin trap, DMPO (120 mM), in 50 mM phosphate buffer, and the reaction was initiated by the addition of 180 mM succinate at room temperature. After 1 h of incubation, the aliquots were subjected to SDS-PAGE and Western blot using an anti-DMPO antibody. As expected, the immobilized nitro spin adduct of SQR was efficiently detected by immunoblotting (Fig. 11A, lane 1) at the 70-kDa subunit of SQR, indicating the formation of SQR-derived protein radical. The detected Western blot signal was entirely dependent on $O_2^-$ generation (as shown by its inhibition by SOD), spin trap, and succinate (Fig. 11A, lanes 2–4), suggesting that $O_2^-$ generation by SQR induced self-inactivation of the enzyme via specific oxidation of 70-kDa subunit, leading to protein radical formation. Replacement of SQR with $S$-glutathionylated SQR subsequently diminished the Western signal of the DMPO adduct of SQR (Fig. 11A, lane 5), supporting the protective role of protein $S$-glutathionylation. It should be noted that superoxide-induced protein radical formation was decreased according to the extent of SQR-SSG enhanced (data not shown).

**DISCUSSION**

Oxidative Impairment of SQR in Post-ischemic Injury—In this study, we have shown that ADP-stimulated state 3 respiration driven by succinate was impaired ($\sim 50\%$ decrease) in mitochondria from post-ischemic hearts (Fig. 1). This was mainly attributed to the impairment of the SQR. In agreement with the notion that mitochondrial membrane depolarization occurs during ischemia/reperfusion injury (40–42, 55), we have observed a significant decrease in the respiratory control ratio in mitochondrial preparations from the post-ischemic myocardium obtained from global ischemia and reperfusion hearts (Fig. 1). The loss of membrane integrity is even obvious in the
risk region with myocardial infarction because of its lack of ADP-stimulated respiration (Fig. 2).

FADH$_2$-dependent oxygen consumption in mitochondrial preparations from post-ischemic myocardium was decreased under the conditions of uncoupling (Fig. 1), indicating the impairment of electron transfer ability from succinate to molecular oxygen by the ETC of the inner membrane. In agreement with this result, decreases in the enzymatic activities of SCR and SQR were detected in tissue homogenates (Fig. 3) from post-ischemic myocardium. Furthermore, no significant changes were detected in the enzymatic activity of QCR in tissue homogenates of the post-ischemic heart. Therefore, marked oxidative injury to SQR occurred in post-ischemic myocardium. This conclusion was further supported by the studies using isolated SCR subjected to ROS exposure (Fig. 4). Similar inactivation of SQR but not QCR was also observed in isolated SCR exposed to high amounts of NO (56). It is likely that QCR is protected from ROS when complexed with SQR.

Protein S-Glutathionylation at the 70-kDa Subunit of SQR—
We have identified and characterized protein S-glutathionylation at the 70-kDa flavin protein of SQR with a combination of immunoblotting and mass spectrometry (Figs. 6 and 7). Furthermore, we have demonstrated the relevance of this event in heart tissue (Fig. 5). As probed by immunoblotting with anti-GSH monoclonal antibody, intrinsic protein S-glutathionylation specific to the 70-kDa subunit was detected in isolated SQR from the bovine heart and tissue homogenates from the rat heart (Fig. 5). In the post-ischemic heart, we observed significant diminution of 70-kDa-derived S-glutathionylation of SQR, presumably because a highly reductive environment is expected under the
conditions of myocardial ischemia. De glutathionylation presumably predisposes the 70-kDa flavin protein to hypersensitivity to oxidative stress induced by ROS during reperfusion injury. This conclusion is based on the following available evidence. (i) Mutations in SdhA (the nuclearly encoded gene for the 70-kDa subunit) have been linked to disorders caused by oxidative stress such as Leigh syndrome (57). (ii) The 70-kDa subunit of SQR has been reported to be the target of oxidative attack by the nitroxylo (HNO) during nitrosative stress in mitochondria (20). This result also implies a likely beneficial role for redox modification with intrinsic GS binding in SQR.

As probed by LC/MS/MS, Cys90 of the 70-kDa precursor is the cysteiny1 residue identified as S-glutathionylated in vitro (Fig. 7). The mature 70-kDa subunit from bovine SQR contains as many as 18 cysteines. The DNA sequence encodes an N-terminal extension containing 43 amino acid residues. This N-terminal extension acts as a mitochondrial import sequence, which has been removed in the mature protein (58). The mature N-terminal sequence is SSAKVSDAIS, which was verified by LC/MS/MS in this study (data not shown). The identified GS-binding domain (77AAFGLSEAGFNTACVTK93) containing Cys90 (Cys47 in mature protein) of bovine SQR is highly conserved throughout the proteins from human (77AAFGLSEAGFNTACVTK85) (where underline indicates Cys90), rat (68AAFGLSEAGFNTACVTK88) (where underline indicates Cys81), yeast (67AAFGLSEAGFNTACVTK67) (where underline indicates Cys80), and Paracoccus denitrificans (35RTACVTK41) (where underline indicates Cys38). From this information, together with the results of this study, we suggest that the Cys90 on the flavoprotein subunit of SQR is one of the regulatory thiolS of mitochondrial SQR, and S-glutathionylation of Cys90 may play a role in antioxidant defense to combat oxidative injury.

It should be noted that various conditions may lead to S-glutathionylation of SQR. For example, different ratio of GSH/GSSG to stimulate oxidative stress conditions can lead to S-glutathionylation reported in the case of complex I (59). Other mediators of S-glutathionylation such as GS-thyl radical or GSNO may mediate protein S-glutathionylation (60). Therefore, site-specific S-glutathionylation would be more convincing if the isolated SQR were subjected to in situ MS analysis. Nevertheless, an attempt to detect the specific cysteine involved in glutathionylation from the isolated SQR was not successful, presumably because of low abundance of GS-binding cysteine in the isolated SQR. Enrichment of GS-binding with GSSG facilitated the detection with LC/MS/MS (Fig. 6, A and C).

The value of current in vitro studies should be recognized as a model. Some glutathionylation is observed when the GSSG/GSH ratio exceeds 0.6 (Fig. 6C). However, the extent of change in high redox ratio because of accumulation of GSSG in mitochondria is unlikely, unless it is a compartmentalized occurrence (redox state from most reducing to most oxidizing: mitochondria > nuclei > cytoplasm > endoplasmic reticulum > extracellular space, see Ref. 19), and re-reduction of GSSG is inhibited. More likely, generation of ROS in the presence of GSH will produce other forms of more reactive glutathionylating species such as GS-OH or GS-thyl radical besides GSSG (see Ref. 23), and these glutathionylating species may react preferentially at a site(s) different from Cys90, which is modified by GSSG.

Normally, mitochondria produce 0.6–1.0 nmol of H2O2/min/mg of protein, accounting for 2% oxygen uptake, under the conditions of state 4 respiration (61). Physiologically, it is likely that intrinsic S-glutathionylation of SQR is an early consequence of mitochondrial oxidative stress. This S-glutathionylation may serve to protect the 70-kDa subunit of SQR from oxidative damage.

Similar observation of enhanced activity resulted from S-glutathionylation was also reported in the case of complex I (62) and sarcoplasmonic reticulum calcium ATPase (63). Therefore, intrinsic S-glutathionylation of SQR should play the protective role against oxidative stress under normal physiological conditions.

Eaton et al. (64) have reported reperfusion of increased S-thiolation of a number of cardiac proteins by 3-fold by loading biotonylated cysteine into the ischemic rat heart. It seems paradoxical that glutathionylation of SQR was detected under the conditions of oxidative stress. However, other examples undergoing glutathionylation under similar conditions of oxidative challenge were also observed in the proteins of actin and caspase-3 (65, 66).

Superoxide Production Mediated by SQR—The generation of superoxide by FADH2 autooxidation has been reported. However, until now there has been no direct evidence supported by EPR. Our detection of the DEPMPO/·OOH adduct by EPR provided a direct measurement of SQR-mediated superoxide generation. The inhibitory effect of TTFa on the detected DEPMPO/·OOH indicated that FADH2 autooxidation partially contributed to the SQR-mediated O2·− generation (−43% under experimental conditions here, see Fig. 8D). The production of O2·− by SQR was minimized under the conditions of enzyme turnover in the presence of Q1 (Fig. 8E).

Based on the x-ray crystal structure (67), Cys90 (Cys47 in the mature protein) is located within the part of the N-terminal β-barrel subdomain (Ala10–Gln61 in the mature protein) of the large FAD-binding domain (residues 10–273 and residues 361–445 in mature protein). Specifically, Cys90 is situated in the hydrophilic pocket with the structure of β-sheet-α-turn-helix-β-turn-sheet-turn-loop (βαβ), suggesting this specific thiol is susceptible to attack by GSSG. The x-ray structure also reveals that Cys90 is near the AMP moiety of FAD (~7.7 Å) where the major catalysis of electron transfer and O2·− production occurs. As demonstrated by EPR spin trapping, the electron transfer coupled with O2·− generation as induced by succinate is partially controlled by reduced FAD cofactor and its FAD-binding protein moiety at the 70-kDa subunit (Fig. 8). Therefore, specific S-glutathionylation at Cys90 seems likely to induce a small conformational change near the FAD-binding site, which might marginally increase the efficiency of electron transfer away from FADH2 to the 2Fe-2S moiety (S1 center), and subsequently decrease the electron leakage under enzyme turnover conditions (Figs. 9 and 10). In post-ischemic injury, deglutathionylation rendered SQR highly susceptible to oxidant stress, presumably because of the
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**Enhancement of Superoxide Generation Ability, Leading to Oxidative Inactivation of SQR.**

Finally, O$_2^-$ generation by SQR also induced self-inactivation with protein radical formation at the 70-kDa Fp subunit as detected by immunospin trapping (Fig. 11). The formation of immobilized free radical(s) on the protein matrix has been implicated as an important molecular event of oxidative damage. The signal of the protein radical at the 70-kDa subunit was subsequently diminished when the Cys$^{90}$ of 70 kDa was S-glutathionylated, further supporting its role in protecting SQR from oxidative attack.

We attempted to map the specific site of protein radical formation with DMPO binding by LC/MS/MS. A doublet protonated molecular ion, (M + 2H)$^{2+}$ = 856.87, of tryptic peptide (649TLNETDCATVPPTIR663) (where underline indicates Cys$^{655}$) was detected, which revealed that Cys$^{655}$ near the C terminus is involved in DMPO binding (data not shown). We did not detect the DMPO adduct with Cys$^{90}$ from LC/MS/MS analysis, which suggests two possibilities. (i) A small conformational change in SQR induced by GS-binding (see above) may render Cys$^{655}$ less susceptible to oxygen-free radical attack. (ii) The instability of the DMPO-peptide adduct after proteolysis may render the dissociation of DMPO from the spin adduct during proteolysis or LC/MS/MS analysis.

In our recent research, we have detected additional glutathionylated cysteine residues when S-glutathionylation of SQR was induced under more drastic conditions (GSSG = 1 mM and GSH = 1 mM, 3 h incubation at 37 °C). These include Cys$^{537}$ and Cys$^{655}$ near the C terminus of 70-kDa flavin protein, identified by the MS/MS spectra of molecular ions of tryptic peptides. 528RVGSVLQEGCKISSLY$^{544}$ (where underline indicates Cys$^{537}$) (at m/z 725.04$^{3+}$) and 651NETDCATVP Insp $^{665}$ (where underline indicates Cys$^{655}$) (at m/z 971.64$^{2+}$). Cys$^{655}$ is associated with protein radical formation with DMPO binding. These data provide evidence of possible involvement of a protein-derived thyl radical in the reactive glutathionylating species that may contribute to S-glutathionylation at the specific cysteine in the presence of GSH.

**Conclusions**—The present studies provide insights regarding the molecular mechanism of oxidative injury and redox modification of SQR in the post-ischemic heart. We have characterized SQR-derived S-glutathionylation and addressed the way this event modulates its electron transfer and superoxide generation activities. The diagram of Fig. 12 illustrates the relationship between protein S-glutathionylation and ROS production, oxidative injury of SQR in mitochondria during I/R. Overproduction of ROS by complexes I and III in mitochondria under the conditions of hypoxia and reoxygenation contributes to the impairment of SQR and mitochondrial dysfunction. Oxidative stress resulting from I/R also disrupts the homeostasis of thiol-related redox state in mitochondria. Specifically, mitochondrial protein thiols (complex II in this study) play a critical role in maintaining this equilibrium. We have detected the reduction of intrinsic S-glutathionylation of SQR in the post-ischemic heart. The enhanced ability of SQR in production of superoxide through deglutathionylation augments the overall magnitude of oxidative injury and mitochondrial dysfunction in the post-ischemic heart.

The mechanism of deglutathionylation is of particular interest because the redox environment is more oxidative during reperfusion, which makes it highly unlikely to occur spontaneously. Specifically, we did not observe significant change with the extent of protein S-glutathionylation during the auto-inactivation of SCR (Fig. 4A) or SQR in the presence of succinate (data not shown). One possible mechanism in triggering deglutathionylation could be related to the mitochondrial glutaredoxin (Grx2). Alternatively, one could surmise that under ischemic conditions (highly reductive), before reperfusion of oxygen, the flavin moiety of SQR becomes fully reduced and transfers electrons to effect reduction of the disulfide bond, yielding SQR-SH plus GSH (analogous to GSSG reductase). Furthermore, the deglutathionylated form of SQR does not necessarily persist during reperfusion because it could undergo...
other types of oxidative modification. Investigation of this event is under progress in our laboratory.

The mechanism addressed here provides a useful concept for understanding the fundamental question of how mitochondrial complex II utilizes its specific redox thiol(s) to address situations of oxidative stress and to regulate its enzymatic functions. Clearly, the major role of S-glutathionylation is to regulate the redox signal that occurred under the conditions of oxidative stress. Furthermore, in vitro studies using isolated enzyme suggested that S-glutathionylation protects the 70-kDa flavin protein from hypersensitivity to oxidative stress, and this event may be to combat oxidative injury by increasing electron transfer efficiency and decreasing electron leakage to molecular oxygen. Recognition of this molecular mechanism is important in understanding the fundamental basis by which oxidants modulate post-ischemic injury caused by mitochondrial dysfunction.

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