Superoxide Generation from Mitochondrial NADH Dehydrogenase Induces Self-inactivation with Specific Protein Radical Formation*

Received for publication, April 12, 2005, and in revised form, August 30, 2005. Published, JBC Papers in Press, September 8, 2005, DOI 10.1074/jbc.M503936200

Yeong-Renn Chen,‡ Liwen Zhang,‡ Kari B. Green-Church‡§, and Jay L. Zweier‡¶

From the ‡Department of Internal Medicine, Davis Heart & Lung Research Institute, Division of Cardiovascular Medicine, the §Department of Molecular and Cellular Biochemistry, College of Medicine, and the ¶Campus Chemical Instrument Center, Proteomics and Mass Spectrometry Facility, The Ohio State University, Columbus, Ohio 43210

Mitochondrial superoxide (O$_2^-$) production is an important mediator of oxidative cellular injury. While NADH dehydrogenase (NDH) is a critical site of this O$_2^-$ production; its mechanism of O$_2^-$ generation in mitochondria is particularly relevant under the physiological conditions of low oxygen tension such as state 4 respiration or certain pathological conditions such as inflammation and ischemia-reperfusion injury (3–5). To understand disease processes associated with oxidative stress, it is necessary to understand the fundamental mechanisms of mitochondrial-derived oxygen-free radical generation.

A decrease in the rate of mitochondrial oxidative phosphorylation can increase the production of superoxide anion radical (O$_2^-$) in the early stages of the electron transport chain (ETC). Two segments of the ETC have been widely hypothesized to be responsible for O$_2^-$ generation in mitochondria (6). One site is located on complex III. At this site, O$_2^-$ production is mediated through the Q-cycle mechanism, in which electron leakage results from the auto-oxidation of ubisemiquinone and reduced cytochrome $b_{566}$ (7–10).

The other site is located on complex I (11). Purified bovine heart complex I (or NADH ubiquinone reductase) contains up to 46 different subunits with a total molecular mass of 980 kDa (12). With the use of chaotropes such as perchlorate, complex I can be resolved into three fractions: a flavoprotein fraction (Fp), an iron-sulfur (Fe-S) protein fraction (lp), and a hydrophobic protein fraction (Hp). The redox centers of complex I that are involved in mediation of two-electron transfer from NADH to ubiquinone include a non-covalent binding Fp, 8 Fe-S centers, and ubiquinone (14, 15).

In addition to the function of electron transfer required for energy transduction in mitochondria, complex I is also responsible for O$_2^-$ generation, which is frequently linked to disease pathophysiology (16). The oxidative damage of complex I has been identified in a number of diseased conditions such as ischemia-reperfusion injury (17–19) and Parkinson’s disease (20, 21).

Two redox components of complex I are logically hypothesized to be involved in the electron leakage for O$_2^-$ generation: one located on the Fp-containing Fp fraction, and one on the Q-binding site that mediates ubiquinone reduction. Based on this hypothesis, reduced FMN semiquinone radical (FMNH$_2$) (22) and ubisemiquinone radical (Q$^\cdot$) can provide the sources of O$_2^-$ generation.

The Fp fraction of complex I contains the enzymatic activity of NADH dehydrogenase (NDH), and can be isolated as a three subunit subcomplex from submitochondrial particles (SMP) (13). Therefore, the Fp-containing three subunit subcomplex is normally termed as NDH. Of the three subunits, the 51-kDa subunit is an NADH-binding subunit and was O$_2^-$ dependent. Alkylation of the cysteine residues of NDH significantly inhibited NDH-DMPO spin adduct formation, indicating involvement of protein thyl radicals.

Mitochondria are the major cellular source of oxygen free radicals (1, 2). The generation of reactive oxygen species (ROS) and free radicals in mitochondria is particularly relevant under the physiological conditions of low oxygen tension such as state 4 respiration or certain pathological conditions such as inflammation and ischemia-reperfusion injury (3–5). To understand disease processes associated with oxidative stress, it is necessary to understand the fundamental mechanisms of mitochondrial-derived oxygen-free radical generation.
Superoxide Generation by Mitochondrial NADH Dehydrogenase

erties are markedly different. It can catalyze NADH oxidation in vitro by a variety of electron acceptors such as ubiquinone-1 (Q$_1$), ferricyanide, menadione, cytochrome $c$, and 2,6-dichlorophenol.

Because of the absence of the Q-binding domain of Hp and most of the iron-sulfur clusters of Ip, NDH provides a simplified and excellent model system to study the molecular mechanism of FMN moieties-mediated O$_2^-$ generation and the role of this O$_2^-$ in inducing oxidative damage to the protein.

It is widely recognized that EPR spin-trapping with DEPMPO (5-diethoxylphosphoryl-5-methyl-1-pyrroline N-oxide) is a sensitive and reliable approach to measure O$_2^-$ production in biological systems. DEPMPO can efficiently trap O$_2^-$ to form a stable adduct, as demonstrated in several enzymatic systems such as eNOS (24), iNOS (25), nNOS (26, 27), xanthine oxidase (28), and leukocyte NADPH oxidase (29).

Immunospin-trapping with a polyclonal antibody against DMPO (5,5-dimethyl-1-pyrroline N-oxide) nitro oxide adduct has developed as a powerful approach to detecting protein radicals resulting from the oxidative attack of ROS (30). The antibody exhibits the advantage of high specificity and high sensitivity detection of protein-derived radicals.

The present investigation was undertaken to address the fundamental mechanism of O$_2^-$ generation by NDH and its effects on the enzyme. We show by EPR spin-trapping that the FMN and the FMN-binding protein moiety in NDH play the primary role in controlling enzyme-mediated O$_2^-$ production. We further observed by immunospin-trapping with anti-DMPO antibody that the 51-kDa subunit of NDH is the site of O$_2^-$-induced protein radicals. By mass spectrometry (MS), these NDH-derived protein radicals were shown to be specifically located at Cys$^{206}$ and Tyr$^{177}$ of the 51-kDa subunit.

MATERIALS AND METHODS

Reagents

Ammonium sulfate, Q$_1$, Zn/Cu superoxide dismutase (SOD), sodium cholate, DPI, $p$-chloromercuribenzoate (CMB), NEM, FMN, $\beta$-nicotinamide adenine dinucleotide phosphate (reduced form, NADPH), and $\beta$-nicotinamide adenine dinucleotide (reduced form, NADH) were purchased from Sigma and used as received. Catalase (bovine liver) was purchased from Roche Diagnostics (Indianapolis, IN). The DEPMPO spin trap was 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.

Preparations of Mitochondrial NDH

Bovine heart mitochondrial NDH was prepared according to the published method with modifications (13). SMP were prepared (31) and used as starting material. 2.5 pounds of trimmed bovine hearts with fat and connective tissues cleaned off were routinely used for SMP preparation. SMP preparation was homogenized with 220 ml of cold water, and then subjected to centrifugation at 95,900 $\times g$ for 1 h. The precipitate obtained was homogenized in cold water to a final volume of 1.2 liters. The pH of SMP homogenate was adjusted to 5.5 with 2 N acetic acid. The mixture was centrifuged for 20 min at 4000 rpm using a Beckman JS 4.2 rotor. The precipitate was then immediately washed with 550 ml of cold water and then homogenized with cold water to a final volume of 400 ml with a protein concentration of about 40 mg/ml. The pH of homogenate was adjusted to 4.8 with 2 N acetic acid. 100% ethanol was added to a final concentration of 9% (v/v). The sample was put into a water bath at 55 °C with constant stirring until it reached 40 °C and stayed at 40 °C for 10 min. The sample was immediately brought to an ice-salt water bath with constant stirring until it reached 4 °C. The homogenate was subjected to ultracentrifugation at 95,900 $\times g$ for 30 min, and the gold/red color supernatant was collected. The crude NDH was concentrated by precipitation with 57% saturation of ammonium sulfate. The precipitate of crude NDH was dissolved in 5 mM potassium phosphate buffer, pH 6.5 (buffer A), and desalted on a Sephadex G-25 column equilibrated with buffer A. The effluent obtained from the Sephadex G-25 column was immediately applied to a DEAE-Sepharose column equilibrated with buffer A. The column was then washed with buffer A until no colored effluent came out. The pure NDH was eluted with buffer A containing 25 mM NaCl. Pure NDH was then concentrated by precipitation with 60% saturation of ammonium sulfate, kept under argon saturation, and stored at $-80$ °C until use. The purity of isolated NDH was examined by SDS gel electrophoresis using a 4–20% gradient of polyacrylamide.

Analytical Methods—Optical spectra were measured on a Shimadzu 2401 UV/VIS recording spectrophotometer. The protein concentration of SMP and NDH was determined by the Lowry method using bovine serum albumin as standard (32). The concentration of Q$_1$ was determined by absorbance spectra from NaBH$_4$ reduction using a millimolar extinction coefficient $\varepsilon_{275-290}$ nm = 12.25 mM$^{-1}$ cm$^{-1}$ (33). The electron transfer activity (ETA) of NDH was assayed by measuring NADH oxidation by Q$_1$ (34). An appropriate amount of NDH was added to an assay mixture (1 ml) containing 50 mM Tris-Cl, pH 8.0, 0.4 mM Q$_1$, and 0.15 mM NADH. The NDH activity was determined by measuring the decrease in absorbance at 340 nm. The specific activity (nmol NADH oxidation/min/mg protein) of NDH was calculated using a molar extinction coefficient $\varepsilon_{340}$ nm = 6.22 mM$^{-1}$ cm$^{-1}$ (34).

Electron Paramagnetic Resonance Experiments

EPR measurements were performed using the EPR Core Facilities at the Davis Heart and Lung Research Institute. Experiments were carried out on a Bruker EMX spectrometer operating at 9.86 GHz with 100 kHz modulation frequency at room temperature. The reaction mixture was transferred to a 50-μl capillary, which was then positioned in the HS cavity (Bruker Instrument, Billerica, MA). The sample was scanned using the following parameters: center field, 3510 G; sweep width, 140 G; power, 20 milliwatt; receiver gain, 2 $\times$ 10$^3$; modulation amplitude, 1 G; time of conversion, 81.92 ms; time constant, 327.68 ms; signal average, 3 scans. The spectral simulations were performed using the WinSim program developed by Duling of NIEHS (35).

Immunoblotting Analysis

The reaction mixture was mixed with the Laemmli sample buffer at a ratio 4:1 (v/v), 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 a nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 10% methanol. Membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TTBS) with 5% dry milk (Bio-Rad). The blots were then incubated overnight with anti-DMPO polyclonal antibody (a gift from Dr. Ronald Mason of NIEHS, Research Triangle Park, NC) at 4 °C. Blots were then washed three times in TTBS, and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG in TTBS at room temperature. The blots were again washed twice in TTBS, twice in TBS, and then visualized using ECL Western Blotting detection reagents (Amersham Biosciences). The signal intensity of blotting was digitalized and quantitated using an AlphaImager™ high performance gel documentation & image analysis system, model 3300 (Alpha Innotech Co. San Leandro, CA).
Mass Spectrometry

The sample of NDH was subjected to SDS-PAGE using 10% polyacrylamide gels. Protein bands on the gel were then stained with Coomassie Blue and subjected to MS measurement.

(a) In-Gel Digestion—Gels were digested with sequencing grade trypsin (Promega) 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 material. After washing twice in 50% methanol/5% acetic acid for several hours, the gel bands dehydrated with acetonitrile were reconstituted with dithiothreitol solution to reduce the cysteines. Iodoacetamide was added to alkylate the cysteines, and the gel was washed again with cycles of acetonitrile and 100 mM ammonium bicarbonate buffer. The gels were then dried using a speed vac. 50 μl of trypsin (20 ng/μl) or chymotrypsin (25 ng/μl) in 50 mM ammonium bicarbonate buffer were added to the dehydrated gel. The gel was set on ice for 10 min for rehydration before the addition of another 20 μl of 50 mM ammonium bicarbonate buffer. The mixture was then incubated at room temperature overnight. The peptides were extracted from the gel using 50% acetonitrile with 5% formic acid several times and pooled together. The extracted pools were concentrated in a speed vac to ~25 μl.

(b) Nano-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 (TM) 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 (TM) system (Dionex, Sunnyvale, CA). Solvent A was water containing 50 mM acetic acid and Solvent B was acetonitrile. A 5-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 Q-TOF system using a gradient of 2–80% B over 48 min, with a flow rate of ~500 nl/min. A total run time was 55 min. The nanospray capillary voltage was set at 3.0 kV and the cone voltage at 48 min, with a flow rate of 500 nl/min. A total run time was 55 min. A total run time was 55 min. A total run time was 55 min.

The experimental spectrum was recorded after signal averaging three scans at room temperature. B, the same as A, except that the enzyme was omitted from the system. C, the same as A, except that the substrate NADH was omitted from the system. D, the enzyme was heated at 70 °C prior to EPR measurement. E, the same as A, except that SOD (0.33 units/μl) was added to the mixture before the reaction was initiated by NDH. F, the same as A, except that NDH was replaced with NADPH. G, the same as A, except that NDH was pretreated with DPI (25 μM) prior to NADH initiation. H, the same as A, except Q1 (400 μM) was included in the mixture prior to the addition of NDH.

signal averaging three scans revealed a multi-line spectrum of a DEPMPO adduct. The spectrum was simulated as a superposition of two isomers of the DEPMPO radical adduct of O$_2$ (DEPMPO/OOH, Fig. 1A, dashed line), based on the hyperfine coupling constants: isomer 1, $a^1_a = 13.14$ G, $a^1_H = 11.04$ G, $a^1_\gamma = 3.46$ G, $a^1_p = 49.96$ G (80% relative concentration); isomer 2, $a^{2}_a = 13.18$ G, $a^{2}_H = 12.59$ G, $a^{2}_\gamma = 3.46$ G, $a^{2}_p = 48.2$ G (20% relative concentration) (36).

The O$_2^-$ generation mediated by NDH absolutely depended on both enzyme and substrate (Fig. 1, B and C). When the purified NDH was subjected to heat denaturation at 70 °C for 5 min, more than 95% of the superoxide generation activity (SGA) was abolished (Fig. 1D), suggesting that ETA is required. The addition of SOD (0.33 units/μl) prior to NADH quenched the signal of DEPMPO/OOH almost completely. Replacement of NADH with the same amount of NADPH yielded a SGA of about 7% of the original (Fig. 1F), demonstrating the substrate specificity for NDH-mediated O$_2^-$ generation. The SGA of NDH was inhibited by DPI (>95% inhibition at 25 μM DPI (Fig. 1G)), suggesting that the FMN-binding moiety was involved in the O$_2^-$ generation. In the presence of various amounts of Q1 as an electron acceptor, the SGA was gradually decreased (data not shown). The maximal reduction (~85% decrease) of SGA was observed at the concentration of 400 μM Q1 used (Fig. 1H).

When the rate of O$_2^-$ production by NDH, as detected by DEPMPO spin-trapping, was graphed versus NADH concentration, it was observed to follow Michaelis-Menten kinetics (Fig. 2). The apparent $K_m$ of

**Superoxide Generation by Mitochondrial NADH Dehydrogenase**

**FIGURE 1.** EPR spin-trapping of O$_2^-$ generated from NDH in the presence of DEPMPO. A, the computer simulation (dashed line) superimposed on the experimental spectrum (solid line) obtained using NDH (0.62 μM), DEPMPO (20 mM), and NADH (0.5 mM) in PBS. The experimental spectrum was recorded after signal averaging three scans at room temperature. B, the same as A, except that the enzyme was omitted from the system. C, the same as A, except that the substrate NADH was omitted from the system. D, the enzyme was heated at 70 °C prior to EPR measurement. E, the same as A, except that SOD (0.33 units/μl) was added to the mixture before the reaction was initiated by NDH. F, the same as A, except that NDH was replaced with NADPH. G, the same as A, except that NDH was pretreated with DPI (25 μM) prior to NADH initiation. H, the same as A, except Q1 (400 μM) was included in the mixture prior to the addition of NDH.
for NADH was estimated to be 0.234 mM based on a Lineweaver-Burk plot (Fig. 2, inset).

The Roles of Redox Centers of FMN and Fe-S Clusters in $\text{O}_2^-$ Production by NDH—The inhibitory effect of DPI on the NDH-mediated $\text{O}_2^-$ production implies that both FMN and the FMN-binding moiety of the 51 kDa subunit play critical roles in controlling $\text{O}_2^-$ generation by NDH (22, 37). To establish the role of FMN cofactor, the FMN (1 $\mu$M) was added to a reaction mixture containing NDH, DEPMPO, and NADH. The $\text{O}_2^-$ production measured by DEPMPO spin-trapping was enhanced by 115%, and by up to 300% when the concentration of FMN was increased to 10 $\mu$M (Fig. 3). In the absence of NDH, however, FMN and NADH induced only trace $\text{O}_2^-$ formation (Fig. 3). Together with the inhibitory effect of DPI and the enhancing effect of FMN, these results imply that mediation of $\text{O}_2^-$ generation is mainly controlled by non-covalently binding FMN and the protein moiety of the FMN-binding site.

NDH contains two Fe-S centers, 4Fe-4S and 2Fe-2S, located on the 51-kDa subunit and the 24-kDa subunit, respectively. The binding motifs are CXXXXCX, C (C, cysteine residue) for 4Fe-4S (38), and CXXXXX$^{\Delta}_{\text{C}}$CXXXC for 2Fe-2S (39). To further establish the essential role of FMN in the SGA activity of NDH, CMB was used to modify the cysteine ligands of the Fe-S clusters (22). The enzyme suffered a 50% loss of ETA without significant loss of its SGA at a CMB concentration of 5 $\mu$M (Fig. 4). However, when 7.5 $\mu$M CMB was added, the ETA of NDH decreased up to 75%, while the NDH-derived SGA was diminished by up to 80% (Fig. 4). More than 90% of both the ETA and SGA were inhibited when the concentration of CMB was increased to 10 $\mu$M (Fig. 4). Complete inhibition of the ETA and SGA was achieved when the dosage of CMB was further increased to 15 $\mu$M. These results imply that both Fe-S clusters are critical for NDH-derived ETA and one specific Fe-S center of NDH is involved in NDH-derived SGA.

Although the partial destruction of the Fe-S cluster partially diminished the NDH-derived SGA, it was expected that the Fe-S cluster should play a secondary role in controlling the catalysis of $\text{O}_2^-$ generation by NDH. Therefore, the inhibitory effect of CMB (7.5 $\mu$M used) on the SGA of NDH should be re-activated by external addition of FMN (10 $\mu$M). As expected, the addition of FMN (10 $\mu$M) to CMB-inhibited NDH increased its $\text{O}_2^-$ generating activity from 20 to 165% (Fig. 5c), thus further confirming the primary role of the FMN cofactor in the SGA of NDH. In the absence of NDH, a trace amount of DEPMPO/OOH ($\sim$10–12%) was detected; this $\text{O}_2^-$ generation was presumably because of auto-oxidation of reduced FMN, forming FMNH.

However, a similar enhancement of SGA by an exogenous supplement of FMN was not observed in the case of NDH subjected to complete inhibition by higher dosage (15 $\mu$M) of CMB. As indicated in Fig. 5, E and F, the addition of FMN (10 $\mu$M) to the CMB-inhibited NDH only increased its SGA to the basal level of non-enzymatic reactions, indicating that a fully or partially active iron-sulfur cluster is required in FMN/NDH-mediated $\text{O}_2^-$ production.

The Role of the FMN-binding Protein Moiety in $\text{O}_2^-$ Production Mediated by NDH—Previous results demonstrated the enhancing effect of FMN cofactor in $\text{O}_2^-$ generation mediated by NDH. It is important to know whether the protein moiety of FMN-binding site is required in this enhancement. To address this question, the NDH was pretreated with DPI (25 $\mu$M), and followed by measuring the catalysis of $\text{O}_2^-$ production in the presence of DEPMPO and NDH. More than 97% of the $\text{O}_2^-$ generation was inhibited (Fig. 6b). The addition of FMN (10 $\mu$M) to the DPI-inhibited NDH only resulted in partial restoration (~15%) of 

**FIGURE 2.** $\text{O}_2^-$ generation from NDH as function of NADH concentration. The system contained NDH (0.62 $\mu$M) and DEPMPO (20 $\mu$M) in PBS. The superoxide generation was initiated with different concentrations of NADH. EPR spectra were recorded after signal averaging three scans and subjected to computer simulation using the parameters described under “Results.” The spin quantitation of each spectrum was obtained by double integration of the simulation spectrum, and plotted against the concentration of NADH. The instrumental settings are described under “Materials and Methods.” Inset, double reciprocal plot.

**FIGURE 3.** The effect of FMN on $\text{O}_2^-$ production by NADH-energized NDH as measured by DEPMPO spin-trapping. The components in the system were NADH (0.62 $\mu$M), FMN (1 $\mu$M and 10 $\mu$M), and DEPMPO (20 $\mu$M) in PBS. NADH (0.5 $\mu$M) was added to initiate $\text{O}_2^-$ generation, and the spectra were collected after signal averaging three 81-s scans. The spin quantitation of each spectrum was obtained as described in the legend to Fig. 2.

**FIGURE 4.** Effect of CMB on the ETA and SGA generation activity (SGA) of NDH dehydrogenase. The mixture containing NDH (0.62 $\mu$M) and various concentrations of CMB in PBS was incubated at room temperature for 5 min. An appropriate amount of CMB-treated enzyme solution was withdrawn and subjected to ETA measurement as described under “Materials and Methods.” For measuring the effect on the SGA of NDH, the experimental approach was the same as that described in the legend of Fig. 1 except that NDH was replaced with CMB-treated NDH.
the NDH-derived SGA (Fig. 6C), implying a critical role for the FMN-binding protein moiety. In a separate experiment, the NDH was subjected to heat denaturation at 70 °C for 5 min, resulting in a 97.5% inhibition of SGA (Fig. 6D). The addition of FMN (10 μM) to heat-denatured NDH could not restore the NDH-derived SGA (Fig. 6E), further confirming the essential role of the FMN-binding protein moiety in NDH-mediated O₂·⁻ generation.

O₂·⁻ Produced by NDH Induces Self-inactivation of the Enzyme via the Formation of NDH Protein Radicals—It has been suggested that a vicious cycle of O₂·⁻-derived H₂O₂ in mitochondria can accelerate inactivation of complex I via the destruction of its iron-sulfur clusters (22). However, a similar mechanism in which O₂·⁻ may induce the oxidative attack of the protein matrix of complex I is also logically hypothesized. NDH provides a simplified model system to test this hypothesis. Incubation of NDH (50 μg/ml) with NADH (1 mM) at room temperature resulted in progressive loss of ETA (Fig. 7). The presence of SOD (1 unit/ml) partially protected NDH from the self-activation induced by O₂·⁻ production (Fig. 7), indicating that the Fp fraction of complex I is susceptible to oxidative stress induced by O₂·⁻. Further protection was seen with addition of catalase (0.2 μg/μl) (Fig. 7), indicating that H₂O₂ formation from O₂·⁻ spontaneous dismutation also contributed to NDH inactivation.

One logical fate for the O₂·⁻ generated in vitro is to react with the protein matrix of NDH, forming protein-derived radicals and subsequently leading to enzyme inactivation. To test this hypothesis, we employed the technique of immunospin-trapping using a polyclonal antibody against DMPO nitrone adduct (anti-DMPO antibody) (30, 40). NDH (0.5 mg/ml) was incubated with a nitrone spin trap, DMPO (100 μM), in PBS buffer, and the reaction was initiated by the addition of 1 mM NADH at room temperature. After a 1-h incubation, the aliquots were subjected to SDS-PAGE and Western blot using an anti-DMPO antibody. As expected, the immobilized nitrone adduct of NDH was efficiently detected by immunoblotting (Fig. 8A, lane 2) at the 51-kDa
Superoxide Generation by Mitochondrial NADH Dehydrogenase

FIGURE 8. Detection of the DMPO adducts of the NDH-derived protein radicals by Western blot using anti-DMPO nitrone adduct polyclonal antibody. A, the reaction mixture contained NDH (0.5 mg/ml, 6.2 μl) and DMPO (100 mM) in 0.1 ml of PBS. NADH (1 mM) was added to initiate the reaction. The reaction was allowed to incubate for 1 h at room temperature, terminated by addition of sample buffer containing 0.4% SDS and 1% β-mercaptoethanol, and then heated at 70 °C for 5 min. Aliquots of 10 μg of protein were subjected to SDS-PAGE and Western blot as described under "Experimental Procedures." In lane 1 and on O2− generation (Fig. 8B, lane 4), indicating that the O2− generation by NDH-induced self-inactivation of the enzyme via site-specific oxidation at the protein matrix of the 51-kDa subunit, leading to protein radical formation. Furthermore, it was observed that pretreatment of NDH (0.5 mg/ml) with DPI (25 μM) substantially diminished (≈70% decrease, n = 3, data not shown) adduct formation, indicating the involvement of FMN cofactor and its FMN-binding protein moiety.

The 51-kDa subunit of complex 1 has been suggested to house reactive/regulatory protein thiols(s), which can respond to oxygen free radicals and induce a redox change (41). It is logical to hypothesize that the critical cysteine(s) of the 51-kDa subunit of NDH should be a potential target for oxidative attack induced by O2− generation. To test this hypothesis, the thiol-blocking reagent, NEM, was used to alkylate the cysteine residues on the surface of NDH. The resultant NEM-NDH was subsequently incubated with NADH in the presence of DMPO, and then subjected to SDS-PAGE and immunoblotting with anti-DMPO antibody. As indicated in Fig. 8C (lane 3), the Western signal of the nitrone adduct of NEM-NDH was significantly inhibited (≈70% inhibition, n = 3) compared with the NDH adduct, implying that the NDH-derived thiol radical is involved in the protein oxidation caused by vicarious cycle of O2− generation.

Involvement of Cys166 and Tyr177 of the 51-kDa Subunit in the DMPO-binding Sites Determined by Mass Spectrometry—To further provide direct evidence for the molecular mechanism of the NDH-derived protein radical induced by O2− attack, it is imperative to determine the location of DMPO binding. The DMPO nitrone adduct of NDH was subjected to SDS-PAGE under reducing conditions. The protein band at 51 kDa was cut out and subjected to in-gel digestion with trypsin and chymotrypsin, respectively, as described under "Materials and Methods," followed by nano-LC MS/MS analysis. The resulting mass spectra acquired from the tryp tic and chymotryptic digests contain ions that correspond in mass to tryptic and chymotryptic peptides of NDH and account for over 81% of the amino acid sequence of NDH.

Because the electrospray spectra are expected to reveal ions that differ in mass by 111 Da between DMPO binding and native peptides (42), the mass spectra from the digests were investigated for the addition of 111 Da to the tryptic or chymotryptic peptides. This mass difference was observed for two tryptic peptides: DMPOC206 (amino acids 200–219, GAGAYICGETALIESIEGK) and DMPOY177 (amino acids 176–184, AYEAGLIGK). The doubly protonated molecular ion (M+2H)2+ of tryptic peptide DMPOC206 was observed at m/z 1062.42, an ion that corresponds in mass to the parent ion (m/z 1006.98 for (M+2H)2+) plus an additional 111 Da. Likewise, the doubly protonated molecule ion of tryptic peptide DMPOY177 was observed at m/z 516.47, an ion that corresponds in mass to an increase of 111 Da compared with that of the parent ion (m/z 460.75 for (M+2H)2+). These data suggest that one DMPO is covalently bound to one of the residues of the NDH 51-kDa subunit in DMPOC206 (residues 200–219) and DMPOY177 (residues 176–184).

To determine which amino acid(s) were covalently linked with the DMPO, the MS/MS spectra of the (DMPOC206)2+ ion of m/z 1062.42 and the (DMPOY177)2+ ion of m/z 516.47 were acquired (Fig. 9). Under the conditions of low energy CID (collision-induced dissociation), both parent ions were fragmented to the single charge state as indicated in the MS/MS spectra of Fig. 9. In both spectra, both y and b ions are observed; these correspond to cleavages along the peptide backbone (43, 44). The y series ions result from C-terminal peptide cleavages, and the b series ions result from N-terminal peptide cleavages.

In the spectrum of the (DMPOY177)2+ ion of m/z 516.97 (Fig. 9A), some of the structurally informative fragment ions, including b2, b3, b4, b5, and b6, are because of the increase of 111 Da, thus allowing unequivocal assignment of the DMPO adduct to the tyrosine 177 residue of the tryptic peptide DMPOY177. It was also observed that another structurally informative ion (y8 in Fig. 9A) with m/z 850.93 lost DMPO binding from the corresponding protonated molecule (theoretical m/z 961.54 for y8).

In the spectrum of the (DMPOC206)2+ ion of m/z 1062.42 (Fig. 9B), the DMPO binding was lost from structurally informative ions, including y14*, y15*, y16*, and b15*. Presumably, this could be because of the weak C–S bond between DMPO and the cysteine residue, which is susceptible to bond breakage during the measurement of MS/MS. However, the determined sequence of this DMPO-binding peptide completely matched the expected sequence (b3-b6 and y1-y13). Together with the result of immunospin-trapping using a thiol-blocked enzyme, NEM-NDH (Fig. 8C), cysteine 206 is unambiguously assigned to be the site of DMPO binding. It is worth noting that we are able to identify two
very weak fragment ions of m/z 1590.00 and 1703.12 (y14 and y15 in TABLE ONE) from the MS/MS spectrum of the (DMPOC206)2/H11001 ion (Fig. 9B). The molecular weight difference between y14 and y13 is 214, corresponding to the cysteine residue attached to one DMPO (TABLE ONE, italics).

DISCUSSION

In the current investigation, we have provided direct evidence for NDH-mediated O2\textsuperscript{−} generation using EPR spin-trapping. With a combination of immunospin-trapping and mass spectrometry, we have demonstrated the relevance of NDH-derived protein radical(s) in the O2\textsuperscript{−}-induced self-inactivation of the enzyme.

Our spin-trapping study with DEPMPO has shown that NDH-mediated O2\textsuperscript{−} generation as induced by NADH is tightly controlled by two important factors, FMN cofactor, and its FMN-binding protein moiety at the 51-kDa subunit. The enhancement of O2\textsuperscript{−} generation by external FMN requires an active protein structure, as shown by the data obtained from the DPI-inhibited enzyme and heat-denatured enzyme (Fig. 6). Although it is not clear if free exchange takes place between the endogenous FMN and exogenously added FMN or not, the involvement of FMN is clear. DPI is an inhibitor that has been used to inhibit the enzymatic activity of several flavoproteins such as eNOS (45), NADPH oxidase (46), and xanthine oxidase (47). Consistent with this study, DPI also significantly inhibited the O2\textsuperscript{−} production catalyzed by these enzymes, presumably because of competitive binding to the protein moiety of the FMN-binding site (48, 49).

Destruction of iron-sulfur centers of NDH by CMB (75% ETA inhibition at 7.5 M CMB in Fig. 4) also inhibited enzyme-mediated O2\textsuperscript{−} generation. However, the SGA of CMB-NDH was increased by the addition of FMN (Fig. 5C). Unlike the inhibitory effect of DPI, CMB directly modified the cysteine residues on the surface of the protein. Presumably, CMB-induced cysteine modification does not destroy the catalytic function of the FMN-binding moiety at the 51-kDa subunit. Therefore, the protein moiety can be actively reconstituted with excess FMN to enhance the SGA of NDH.

This SGA enhancement by an exogenous supplement of FMN was not observed in NDH subjected to complete destruction of its Fe-S cluster by a higher concentration of CMB (Fig. 5, E and F). Presumably, the iron-sulfur cluster plays the role of an electron transfer mediator of.
Superoxide Generation by Mitochondrial NADH Dehydrogenase

The reduced flavin mononucleotide (FMNH₂) to generate FMNH⁺ and subsequent O₂⁻.

Furthermore, the results from the CMB-inhibited enzyme imply that one of the Fe-S clusters in NDH is likely to be involved in the O₂⁻ generation catalyzed by NDH. This is likely to occur at the 4Fe-4S center of the 51-kDa subunit because of its higher redox potential (E°₉₋ = -250 mV) compared with that of the 2Fe-2S (E°₉₋ = -370 mV) center of the 24-kDa subunit. Investigation of the detailed mechanism of electron leakage via the iron-sulfur clusters of NDH is currently in progress. It has been proposed that the binding domains for NADH, FMN, and the 4Fe-4S cluster are arranged in that linear order in the sequence from the N to the C terminus of the 51-kDa subunit (38). As proposed in the Fig. 10, FMN is considered to be the immediate oxidant of NADH. It can take two electrons at a time, presumably via a hydride transfer, forming a reduced flavin mononucleotide, FMNH₂. FMNH₂ then releases one electron to the one-electron acceptor of the 4Fe-4S cluster, forming FMNH⁺, which is presumably the source of O₂⁻ (22).

Although the formation of FMNH⁺ required for O₂⁻ production have been widely proposed (22, 60), it still lacks a direct EPR evidence for FMNH⁺. The use of direct EPR to detect transient free radical formation of FMNH⁺ under the conditions of enzyme turnover is under investigation.

One should not rule out the possibility that both electrons are transferred sequentially from the 4Fe-4S cluster to oxygen. The demonstrated need of FMN would arise only because the very first reaction catalyzed by the enzyme is an obligatory hydride transfer from NADH, converting FMN to FMNH₂. Single electrons are then transferred to the 4Fe-4S cluster and from there to oxygen, in the absence of any other acceptor.

Although we have provided substantial evidence for the exact site and mechanism of O₂⁻ production from the flavin site of purified NDH, we do not know whether the same reaction takes place in in vivo mitochondria. Several research groups have proposed that in intact mitochondria and submitochondrial particles, most of the O₂⁻ may be generated around the iron-sulfur N2/ubiquinone site, the last site in the electron transport chain within complex I (50–53). This issue requires further investigation.

The 51-kDa subunit is one of the nuclear encoded subunits of bovine complex I. The DNA sequence encodes an N-terminal extension containing 20 amino acid residues (amino acid residues 1–20 of Fig. 11). This N-terminal extension acts as a mitochondrial import sequence, which has been removed in the mature protein. Therefore, the mature N-terminal sequence is SGDTTAPKKT and the calculated molecular mass of the mature protein is 48,499.4 Da (12, 38, 54).

There is no direct evidence to show the exact region of the FMN-binding domain at the 51-kDa subunit. However, based on the sequence analysis, Walker has proposed that the region of FMN binding is most likely located in the highly conserved glycine rich sequence encompassing amino acids 200–254 (Fig. 11) (38, 54). This region is situated downstream of the proposed substrate/NADH-binding domain (residues 81–119) and upstream of the Fe-S-binding domain (residues 379–425) (Fig. 11).

As probed by immunospin-trapping, the 51-kDa subunit was specifically involved in the protein radical formation induced by the vicious cycle of O₂⁻ production, leading to self-inactivation (Fig. 8). Cys²⁰⁶ was further identified by LC/MS/MS to be the site of oxidative attack (Fig. 9B). Importantly, Cys²⁰⁶ is located within the proposed FMN-binding domain where the major catalysis of O₂⁻ production occurs. The bovine protein has 12 cysteine residues, but only 5 of them are conserved. The first conserved cysteine is Cys²⁰⁶, which is separated from the others by 172 residues. The four remaining conserved cysteine residues are involved in the ligands of the [4Fe-4S] cluster. Therefore, the biological process of O₂⁻ production at the 51-kDa subunit appears to be dependent on the accessibility of the critical cysteine residue at position ²⁰⁶.

### Table One

| Δm between yn & yn-1 | Fragment ion | Measured m/z | Sequence | Measured m/z | Fragment ion | Δm between bn & bn-1 |
|----------------------|--------------|--------------|----------|--------------|--------------|---------------------|
|                      | Gly          |              |          |              |              |                     |
|                      | Ala 129.09   | b2           |          |              |              |                     |
|                      | Gly 186.12   | b3           |          |              |              |                     |
|                      | Ala 257.16   | b4           |          |              |              |                     |
|                      | Ala 275.16   | b5           |          |              |              |                     |
|                      | Tyr 420.25   | b6           |          |              |              |                     |
|                      | Ile 533.35   |              |          |              |              |                     |
|                      | b15-DMPO     | 1591.95      |          |              |              |                     |
| y16-DMPO             | 1755.06      |              |          |              |              |                     |
| y15⁺                  | 1703.12      |              |          |              |              |                     |
| y15 -DMPO            | 204.16       |              |          |              |              |                     |
| 214.16 (103 + 111)   | y14          | 1590.00      | Cys²⁰⁶   |              |              |                     |
| y14 -DMPO            | 1478.86      |              |          |              |              |                     |
| 186.07               | y13          | 1375.84      | Gly      |              |              |                     |
|                      |              | Gly          |          |              |              |                     |
|                      |              | Glu          |          |              |              |                     |
| 129.05               | y11          | 1189.77      | Glu      |              |              |                     |
| 101.07               | y10          | 1060.72      | Thr      |              |              |                     |
| 71.04                | y9           | 959.65       | Ala      |              |              |                     |
| 113.10               | y8           | 888.61       | Leu      |              |              |                     |
| 113.09               | y7           | 775.51       | Ile      | 1478.86      | b15-DMPO     |                     |
| 129.07               | y6           | 662.42       | Glu      |              |              |                     |
| 87.04                | y5           | 533.35       | Ser      |              |              |                     |
| 113.09               | y4           | 446.31       | Ile      |              |              |                     |
| 129.06               | y3           | 333.22       | Glu      |              |              |                     |
| 57.02                | y2           | 204.16       | Gly      |              |              |                     |
|                      | y1           | 147.14       | Lys      |              |              |                     |

a Bold, structurally informative ions and their measured m/z for Cys*.
b Cys*, DMPO-binding cysteine.
The relevance of Cys\textsuperscript{206} to the oxidative damage of complex I is that it may play the unique role of the reactive thiol in the 51-kDa subunit. Taylor et al. (41) have employed a thiol-specific probe and proteomic approach to examine NADH dehydrogenase. Both the 51-kDa and 75-kDa subunits of complex I were implicated as hosts of the redox thiol(s). In our recent research progress, Cys\textsuperscript{206} has been observed to be the major site involved in the protein S-glutathiolation of NDH under conditions of oxidative stress.\textsuperscript{3} Therefore, we conclude that the identified Cys\textsuperscript{206} is the critical thiol of the NDH 51-kDa subunit of complex I.

LC/MS/MS also identified an additional amino acid, Tyr\textsuperscript{177}, that is involved in the formation of immobilized nitrone adduct. Tyr\textsuperscript{177} is not located within the proposed FMN-binding domain, but is between the proposed regions of NDH binding and FMN binding. However, Tyr\textsuperscript{177} is conserved among the proteins from bovine heart, Paracoccus denitrificans and Neurospora crassa (38). The detected protein-derived tyrosyl radical could be derived by thermodynamic electron transfer from the thyl radical to a carbon-centered radical, forming a secondary protein-bound tyrosyl radical (see Equations 1 and 2 below), as reported by Prütz et al. (55). Because chemical modification with NEM alkylation abolished nearly 70% of the signal intensity detected by immunoblotting (Fig. 8C), this suggests that the cysteinyl radical is primary. Currently, it is not clear whether intramolecular pathways are involved in producing the tyrosyl radical on NDH because of the lack of a high resolution x-ray structure. It is likely that a radical equilibrium was established between the thyl radical and the tyrosine residue as seen in the case of the inactivation of xanthine oxidase by oxidative attack (see Equation 3) (56). A similar situation was also observed in the case of bovine serum albumin (57) and cytochrome c oxidase (58, 59) subjected to oxidative attack.

\[
\begin{align*}
\text{CysNDH} + \text{O}_2^- + \text{H}^+ & \rightarrow \text{SCysNDH} + \text{H}_2\text{O}_2 \\
\text{SCysNDH} + \text{TyrNDH} & \rightarrow \text{CysNDH} + \cdot\text{OTyrNDH} \\
\text{SCysNDH} & \rightleftharpoons \cdot\text{OTyrNDH}
\end{align*}
\]

In our recent investigation using isolated complex I from the bovine heart, we observed that \text{O}_2\textsuperscript{2-} generation was also catalyzed by intact complex I in the presence of NDH, as seen in current studies of NDH. However, the efficiency of complex I-mediated \text{O}_2\textsuperscript{2-} production is \sim 40% lower than that by NDH under the same assay conditions (data not shown). Presumably both Ip and Hp fractions of intact complex I can stabilize the electron transfer and subsequently reduce electron leakage. Consistent with the results obtained from NDH, the \text{O}_2\textsuperscript{2-} generation by complex I can be inhibited by DPI (95% inhibition at 125 \mu M of DPI; data not shown). Furthermore, exogenous FMN significantly increased the activity of \text{O}_2\textsuperscript{2-} production by CMB-inhibited complex I (from 53 to 107%; data not shown). Likewise, \text{O}_2\textsuperscript{2-} production induced self-inactivation of the enzymatic activity of complex I with the formation of protein radicals at the 51-kDa subunit as seen in the case of NDH (data not shown). Specific amino acid residues involved in the DMPO binding were identified to be Cys\textsuperscript{206} and Tyr\textsuperscript{177}. Therefore, the results addressed in our current studies are relevant to the intact complex I, and especially pertinent to the function of the Fp portion of complex I.

In conclusion, the present studies provide the molecular mechanism of NDH mediation of \text{O}_2\textsuperscript{2-} production and the way this event induces self-inactivation of the enzyme. The mechanism addressed here provides a useful concept for understanding the fundamental question of how mitochondrial complex I utilizes FMN cofactor and its FMN-binding protein moiety to modulate oxygen free radical production. As clearly implicated by this study, the major damage caused by oxygen free radicals is protein oxidation via formation of the cysteinyl and tyrosyl radicals, which is perhaps involved in the disease pathogenesis related to mitochondrial dysfunction. Defective mitochondrial complex I has been observed in a wide variety of diseases such as ischemia-reperfusion injury and neurodegeneration. Oxidative impairment by ROS is widely accepted as the important causative factor. Recognition of the molecular mechanism addressed in this work is important in understanding the fundamental basis by which oxidants modulate mitochondrial function in a variety of diseases associated with inflammation and oxidant toxicity.

Acknowledgments—We thank Drs. Chang-An Yu and Linda Yu (Oklahoma State University, Stillwater, OK) for valuable advice and Dr. Juan Zhu’s assistance in the isolation of NDH from bovine heart at the beginning stage of this work and Rhonda Pitsch and Nan Kleinholz for assistance in mass spectrometry.
Superoxide Generation by Mitochondrial NADH Dehydrogenase

REFERENCES

1. Baha, S., and Robinson, B. H. (2000) Trends Biochem. Sci. 25, 502–508
2. Turrens, J. F. (2003) J. Biol. Chem. 278, 353–344
3. Zweier, J. L., Flaherty, J. T., and Weisfeldt, M. L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1404–1407
4. Zweier, J. L., Kuppusamy, P., Williams, R., Rayburn, B. K., Smith, D., Weisfeldt, M. L., and Flaherty, J. T. (1989) J. Biol. Chem. 264, 18890–18895
5. Ambrozio, G., Zweier, J. L., Duilio, C., Kuppusamy, P., Santoro, G., Elia, P. P., Tritto, I., Cirillo, P., Condorelli, M., Chiariello, M., et al., Williams, R., Rayburn, B. K., Smith, D., Weisfeldt, M. L., and Flaherty, J. T. (1993) J. Biol. Chem. 268, 18532–18541
6. Cadenas, E., Boveris, A., Ragan, C. I., and Stoppani, A. O. (1977) Arch. Biochem. Biophys. 180, 248–257
7. Turrens, J. F., Alexandre, A., and Lehninger, A. L. (1985) Arch. Biochem. Biophys. 237, 408–414
8. Nohl, H., and Jordan, W. (1986) Biochem. Biophys. Res. Commun. 138, 533–539
9. Zhang, L., Yu, L., and Yu, C. A. (1998) Arch. Biochem. Biophys. 353, 52–56
10. Sanders, S. P., Zweier, J. L., Kuppusamy, P., Harrison, S. J., Bassett, D. J., Gabrielion, E. W., and Sylvester, J. T. (1993) J. Clin. Invest. 91, 46–52
11. Zweier, J. L., Fearnley, I. M., Shannon, R. J., Hirst, J., and Walker, J. E. (2003) J. Biol. Chem. 278, 35336–35339
12. Benboubetra, M., and Harrison, R. (1998) Free Radic. Res. Commun. 27, 457–474
13. Prutz, W. A., Butler, J., and Tordo, P. (1995) J. Biol. Chem. 270, 118–127
14. Roepstorff, P., and Fohlman, J. (1984) Biomed. Mass Spectrom. 11, 601
15. Bovina, C., D'Aurelio, M., Fato, R., Formiggini, G., Genova, M. L., Giuliano, G., Merlo Pich, M., Paolucci, U., Parenti Castelli, G., and Ventura, B. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 159–170
16. Andreeva, I. A., Mayer, S. J., and Jones, O. T. (1998) FEBS Lett. 430, 113–117
17. Hirst, J., Carroll, J., Fearnley, I. M., Shannon, R. J., and Walker, J. E. (2003) J. Biol. Chem. 278, 35336–35339
18. Biemann, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
19. Redfearn, E. R., and Whittaker, P. A. (1966) Biochemistry 4, 755–767
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
21. Petterson, A. G., and Kastin, A. J. (1986) J. Biol. Chem. 261, 11936–11943
22. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
23. Roepstorff, P., and Fohlman, J. (1984) Biomed. Mass Spectrom. 11, 601
24. Anderson, R. F., Hille, R., and Patel, K. B. (1995) J. Biol. Chem. 270, 5263–5268
25. Biemann, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
27. Petterson, A. G., and Kastin, A. J. (1986) J. Biol. Chem. 261, 11936–11943
28. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
29. Hirst, J., Carroll, J., Fearnley, I. M., Shannon, R. J., and Walker, J. E. (2003) J. Biol. Chem. 278, 35336–35339
30. Mason, R. P. (2004) Free Radic. Biol. Med. 36, 1214–1223
31. Vinogradov, A. D., and King, T. E. (1979) Methods Enzymol. 55, 118–127
32. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
33. Petterson, A. G., and Kastin, A. J. (1986) J. Biol. Chem. 261, 11936–11943
34. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
35. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
36. Petterson, A. G., and Kastin, A. J. (1986) J. Biol. Chem. 261, 11936–11943
37. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
38. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
39. Petterson, A. G., and Kastin, A. J. (1986) J. Biol. Chem. 261, 11936–11943
40. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
41. Petterson, A. G., and Kastin, A. J. (1986) J. Biol. Chem. 261, 11936–11943
42. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
43. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
44. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
45. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
46. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
47. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
48. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
49. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
50. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
51. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
52. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
53. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
54. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
55. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
56. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
57. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
58. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
59. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111
60. Biemar, K. (1988) Biomed. Environ. Mass Spectrom. 16, 99–111