Available online at www.sciencedirect.com

SCIENCE DIRECT

Free Radical Biology & Medicine 38 (2005) 969–976

www.elsevier.com/locate/freeradbiomed

Original Contribution

Identification of the myoglobin tyrosyl radical by immuno-spin trapping and its dimerization

Charles D. Detweiler[a], Olivier M. Lardinois[a], Leesa J. Deterding[b], Paul R. Ortiz de Montellano[c], Kenneth B. Tomer[b], Ronald P. Mason[a,*]

[a]*Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, National Institutes of Health, PO Box 12233, MD F0-01, Research Triangle Park, NC 27709, USA*

[b]*Laboratory of Structural Biology, National Institute of Environmental Health Sciences, National Institutes of Health, PO Box 12233, MD F0-03, Research Triangle Park, NC 27709, USA*

[c]*Department of Pharmaceutical Chemistry, University of California at San Francisco, 600 16th Street, San Francisco, CA 94143-2280, USA*

Received 27 September 2004; revised 20 December 2004; accepted 23 December 2004
Available online 13 January 2005

Abstract

5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) spin trapping in conjunction with antibodies specific for the DMPO nitrone epitope was used on hydrogen peroxide-treated sperm whale and horse heart myoglobins to determine the site of protein nitrone adduct formation. The present study demonstrates that the sperm whale myoglobin tyrosyl radical, formed by hydrogen peroxide-dependent self-peroxidation, can either react with another tyrosyl radical, resulting in a dityrosine cross-linkage, or react with the spin trap DMPO to form a diamagnetic nitrone adduct. The reaction of sperm whale myoglobin with equimolar hydrogen peroxide resulted in the formation of a myoglobin dimer detectable by electrophoresis/protein staining. Addition of DMPO resulted in the trapping of the globin radical, which was detected by Western blot. The location of this adduct was demonstrated to be at tyrosine-103 by MS/MS and site-specific mutagenicity. Interestingly, formation of the myoglobin dimer, which is known to be formed primarily by cross-linkage of tyrosine-151, was inhibited by the addition of DMPO.
Published by Elsevier Inc.

Keywords: Antibodies; DMPO; Myoglobin tyrosyl radical; Western blot; Dityrosine; Free radicals

Introduction

Previously, we reported a new approach to spin trapping that uses antibodies to detect 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) nitrone adducts, such as Mb-DMPO [1]. This new approach allows for the detection of many protein radicals using Western blot and ELISA techniques. Unfortunately, assignment of the radical species detected using this immunological approach is problematic. Here we have attempted to solve this problem by employing such techniques as protein modification, site-directed mutagenesis, and mass spectrometric analysis in tandem with our immunological approach, using myoglobin (Mb) as a model.

Ferric heme proteins with peroxide-like activity such as the met form of myoglobin and hemoglobin reduce hydrogen peroxide to water with the concomitant formation of a ferryl-oxo heme and free radicals centered on tyrosine, cysteine, tryptophan, and, in the case of hemoglobin, histidine [2]. The ferryl-oxo heme species is relatively stable at room temperature, but over time, the ferryl species undergoes an autoreduction that regenerates the ferric enzyme and an additional free radical centered on the protein [3]. The tryptophan-centered free radical [4] reacts with oxygen to form a peroxyl radical, which oxidizes external substrates including glutathione and arachidonic acid [5]. Cross-links of sperm whale myoglobin have been detected between two tyrosine residues [6,7] and also between tyrosine-103 and the heme [8,9]. Active site variants of myoglobin, such as sperm whale and horse myoglobins [10], as well as site-directed

* Corresponding author. Fax: (919) 541 1043.
E-mail address: mason4@niehs.nih.gov (R.P. Mason).

0891-5849/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.freeradbiomed.2004.12.031

mutants of myoglobin [11], have been utilized to identify the location of these tyrosyl radicals and their radical adducts. We report here a study that demonstrates that the antibodies raised against the DMPO nitrone epitope specifically bind to the DMPO nitrone adducts formed from the myoglobin tyrosyl-103 radical and that DMPO inhibits tyrosine dimer formation.

Experimental procedures

Preparation and expression of site-directed mutants

The mutant sperm whale myoglobin proteins were expressed, purified, and oxidized to the met form as described previously [3,7]. The far ultraviolet circular dichroism spectra of wild-type and mutant sperm whale myoglobin proteins at pH 6.8 were fully superimposable (data not shown). In all mutants examined, the observed UV-visible spectra were nearly identical to those of the native protein, indicating that changes to the structure in the vicinity of the heme iron were minimal (data not shown). When required, the metmyoglobin concentration was determined from the Soret maximum at 409 nm ($\varepsilon_{metMb,\ 409}$ = 157 mM^{-1} cm^{-1}) [12].

Preparation of iodinated myoglobin

Horse heart myoglobin (1 mg/ml) (USB Corp., Cleveland, OH, USA) was reacted with sodium iodide (25 mM) using an IODO Beads Iodination Reagent (Pierce Chemical Co., Rockford, IL, USA) and following the commercial protocol. After iodination, the myoglobin was dialyzed 2× for 1 h against 1000× excess buffer.

Spin trapping

Recombinant sperm whale myoglobin (Sigma, St. Louis, MO, USA) and horse heart myoglobin (USB Corp.) were reacted with hydrogen peroxide in the presence of DMPO (Alexis Biochemicals, San Diego, CA, USA). Spin trapping reactions used for mass spectrometric analysis were reacted in ammonium bicarbonate buffer (0.05 M, pH 7.8). All other reactions were performed in sodium phosphate buffer (0.1 M, pH 7.4), allowed to proceed for 2 h at 37°C, and then stopped by adding 200 U/ml catalase.

Protein staining

Staining was performed subsequent to SDS-PAGE and electrophoretic transfer onto a nitrocellulose membrane using a NuPAGE system (Invitrogen Corp., Carlsbad, CA, USA) according to the commercial protocol. Protein bands were stained with 2% Ponceau S in 30% trichloroacetic acid for 20 min and then rinsed with deionized water. Digital images were made of the protein bands on the nitrocellulose membrane using Adobe PhotoShop (Adobe Systems, San Jose, CA, USA).

Western blot

The nitrocellulose membrane used for protein staining was destained overnight in deionized water to remove the Ponceau S. Nonspecific sites on the membrane were blocked with blocking solution (2.5% BSA, 2.5% casein, in 10 mM Tris-buffered saline, pH 7.4). The membrane was washed 1× in wash buffer (0.05% BSA, 0.05% casein, 0.05% Tween 20 in 10 mM Tris-buffered saline, pH 7.4) and then incubated 90 min with 1:5000 anti-DMPO rabbit serum dissolved in wash buffer. After another 4×wash, the membrane was incubated 60 min with 1:5000 anti-rabbit IgG conjugated to alkaline phosphatase (Pierce Chemical Co., Rockford, IL, USA) dissolved in wash buffer. The membrane was washed another 4× to remove all unbound antibodies. Positive samples were detected using an enhanced CDP-Star (Roche Applied Science, Indianapolis, IN, USA) solution [250 μM CDP-Star, 0.1 mg/ml Nitro-Block II (PE Biosystems, Bedford, MA, USA) in 10 mM Tris-buffered saline, pH 9.6]. The luminescent protein bands were captured on X-ray film and digitized using Adobe PhotoShop (Adobe Systems).

Electrospray mass spectrometry

A Micromass Q-Tof Ultima Global and a Micromass Q-Tof Micro (Altrincham, UK) hybrid tandem mass spectrometer were used for the acquisition of the electrospray ionization (ESI) mass spectra and tandem mass spectra. These instruments are equipped with a nanoflow electrospray source and consist of a quadrupole mass filter and an orthogonal acceleration time-of-flight mass spectrometer. The needle voltage was ~3000 V and the collision energy was 4.0–10 eV for the MS analyses. For the MS/MS experiments, a parent ion was selected with the first mass analyzer and transmitted into a collision cell where fragmentation was induced by collision with argon atoms. The collision energy used for these experiments was 30 eV. The resulting fragment ions were detected with the second mass analyzer. In this type of experiment, only ions resulting from fragmentation of the selected parent ion were observed. Data analysis was accomplished with a MassLynx data system and MaxEnt deconvolution software supplied by the manufacturer.

Samples for flow injection analyses were infused into the mass spectrometer at ~300 nl/min using a pressure injection vessel. For the LC/MS and LC/MS/MS analyses, an Agilent Cap1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of a micro-vacuum degasser, binary pumps, and a microautosampler was used to deliver the gradients. Aliquots of 8 μl were injected and a linear gradient of 5–95% acetonitrile (0.1% formic acid) over 45 min was used for the chromatographic separations. The