Non-covalent Modulation of pH Dependent Reactivity of a MnSalen Cofactor in Myoglobin with Hydrogen Peroxide

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EXPERIMENTAL METHODS

Materials

Deionized water (DI) was purified by a Millipore (Bedford, MA) water purifier. All HPLC purified oligionucleotide primers (50 nmol scale) were ordered from IDT Technologies (Alameda, CA). Polymerases were purchased from Stratagen (San Diego, CA). Restriction enzymes were purchased from Gibco (Gaithersburg, MD) or New England Biolabs (Beverly, MA) DpnI was obtained from Stratagen. All organic solvents and Escherichia strains were obtained from Novagen (Madison, WI). Bacto-tryptone, and yeast extract were purchased from Fisher Scientific (Pittsburgh, PA). Plasmid purification kits were purchased from Qiagen (Valencia, CA). Sephacryl S-100 high resolution, DEAE-Sepharose CL-6M, and Sephadex G-75 size elusion materials were purchased from Biotech Pharmacia. Isopropyl-ß-D-thiogalactopyanoside was purchased from Diagnostic Chemicals Limited. Potassium bicarbonate was purchased from Malllinckrodt. Acetone, hexane, methylene chloride, ethyl acetate, calcium chloride, Celite, N,N-dimethylformamide, acetonitrile, EDTA disodium salt, lithium bromide, anhydrous magnesium sulfate, potassium phosphate monobasic, and sodium chloride and potassium hydroxide were purchased from Fisher. Phenylmethyl sulfonyl fluoride (PMSF) and Ampicillin sodium salt and were purchased from United States Biochemical. Dithiothreitol (DTT) and d-aminolevulinic acid was purchased from Sigma. Thioanisole and ABTS were purchased from Acros. Phenyl methyl sulfoxide and H_2O_2 (30% in water) were purchased as the racemic mixture from Aldrich. All other analytes and standards, including single enantiomers of optically active compounds, were purchased from Aldrich and used as is without further purification.

Protein Expression and Biocatalysts Preparation

Introduction of mutations at the desired locations in the Mb scaffold was accomplished via quick change mutagenesis and the variant proteins were expressed and purified as reported previously.[1-3] Recombinant sperm whale myoglobin with mutations T39C/L72C was expressed and isolated from *E. coli*, subjected to heme extraction and incorporated with Mn salen via covalent attachment as reported previously.[1] PCR based site directed mutagenesis was performed on the T39C/L72C Mb template to produce apo-

Mb(T39C/L72C/H64F) and Mb(T39C/L72C/H64R) mutants. The successful incorporation of the desired site direct mutation was verified by DNA sequence analysis and ESI-MS.

Generation of the heme free *apo* variants was accomplished via a combination of the methods of Teale[4] and Fisher.[5] Addition of a solution of **1** in dimethylsulfoxide to the apo Mb variant resulted in 100% conversion to the dually anchored MnSalen artificial biocatalysts for all mutants as assessed by ESI mass spectrometry.

Characterization of Biocatalysts 1•Mb(H64X) (X= H, F or R)

Electro-spray ionization mass spectroscopy (ESI-MS) measurements were obtained using a Quattro II mass spectrometer (MicroMass) with 4000 molecular weight resolution maintained by the Micro Analysis Lab at the University of Illinois. Mass scale was calibrated with myoglobin. Samples were prepared in 50 mM NH_4OAc pH 5.1 as described for general sulfoxidation mixed with 0.02% formic acid and run as a single injection with a continuous solvent feed of acetonitrile (ACN). UV-visible absorption spectra were obtained on a Varian Cary 3E spectrophotometer at 10 °C.

For experiments to determine how the **1**•Mb is changed during reaction with H_2O_2 and to determine the location of any oxidation events on the catalyst, 180 µL of 0.13 mM **1**•Mb in 50 mM NH_4OAc pH 5.1 was reacted with 10 µL of 100 mM H_2O_2. After 1-2 hours, the buffer was exchanged by size exclusion chromatography and the sample reconcentrated by ultra filtration.

Apo-Mb(T39C/L72C): UV/Vis: ?max (e $mM^{-1}cm^{-1}$), 280 nm (18.34); ESI MS: 17324.0 ± 2.0 Da. calcd: 17323.0

1•Mb: UV/Vis: ?max (e $mM^{-1}cm^{-1}$), 280 (33.94) and 292 (34.1)nm ESI MS: 17794.0 ± 2.0 Da. calcd: 17794.4.

Apo-Mb(H64F): UV/Vis: ?max (e $mM^{-1}cm^{-1}$), 280 nm (18.34); ESI MS: 17331.0 ± 2.0 Da. calcd: 17333.1

1•Mb(H64F): UV/Vis: ?max (e $mM^{-1}cm^{-1}$), 280 (33.94) and 292 (34.1)nm ESI MS: 17804.0 ± 2.0 Da. calcd: 17804.5.

Apo-Mb(H64R): UV/Vis: ?max (e $mM^{-1}cm^{-1}$), 280 nm (18.34); ESI MS: 17346.0 ± 2.0 Da. calcd: 17342.1

1·Mb(H64R): UV/Vis: ?max (e $mM^{-1}cm^{-1}$), 280 (33.94) and 292 (34.1)nm ESI MS: 17815.0 ± 2.0 Da. calcd: 17813.5.

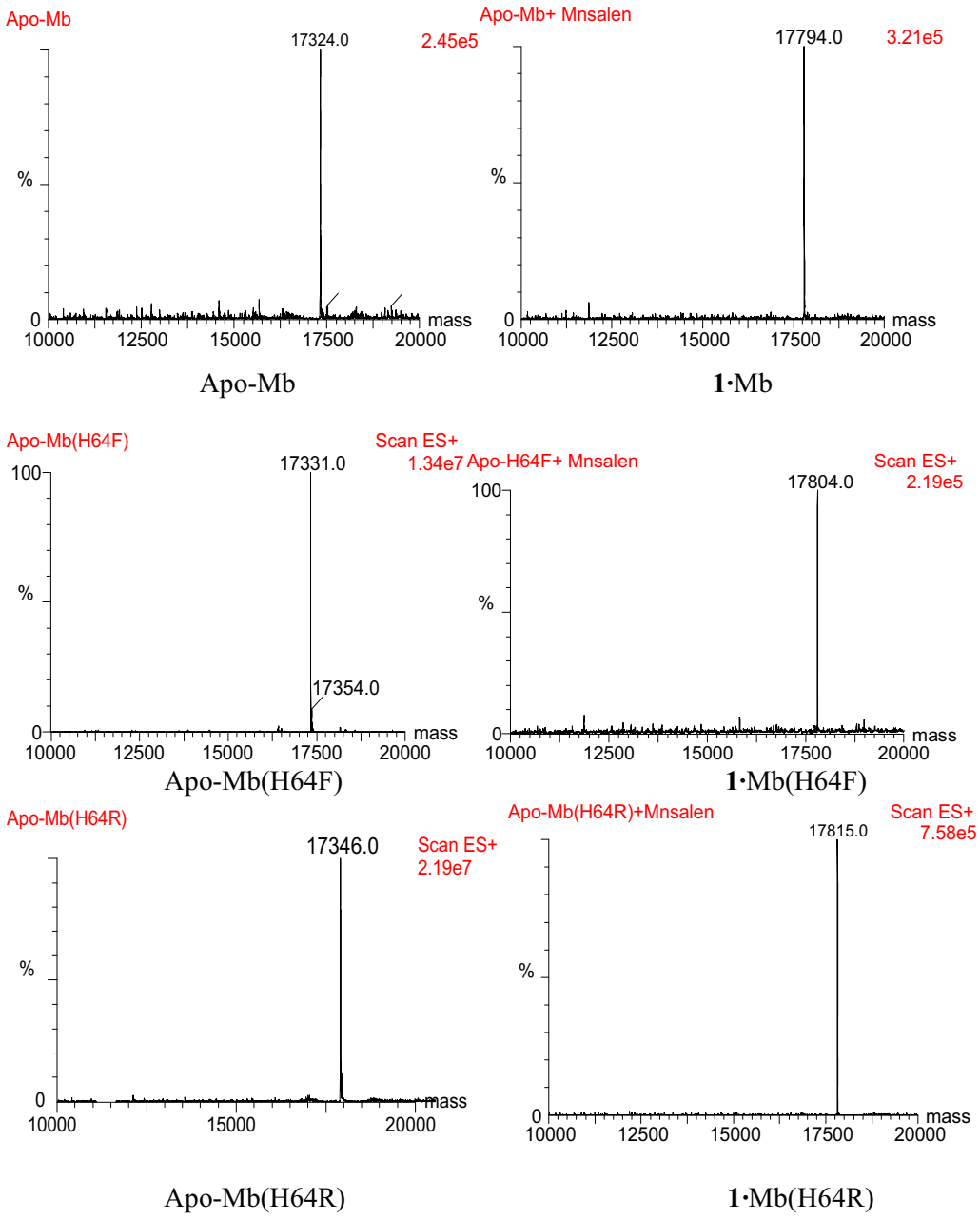
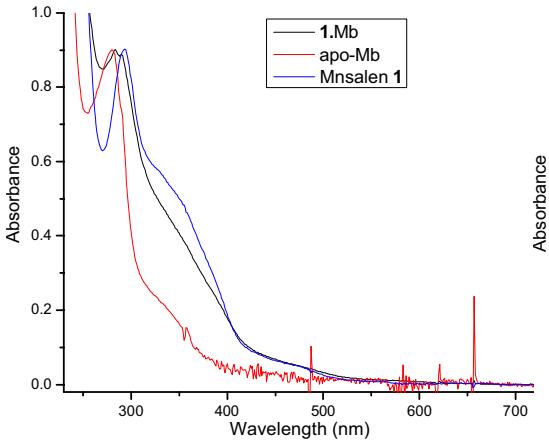
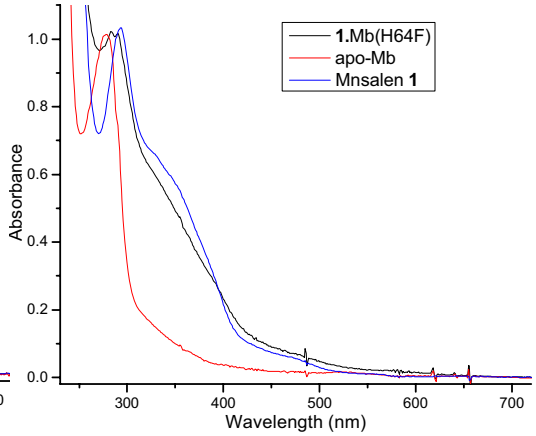


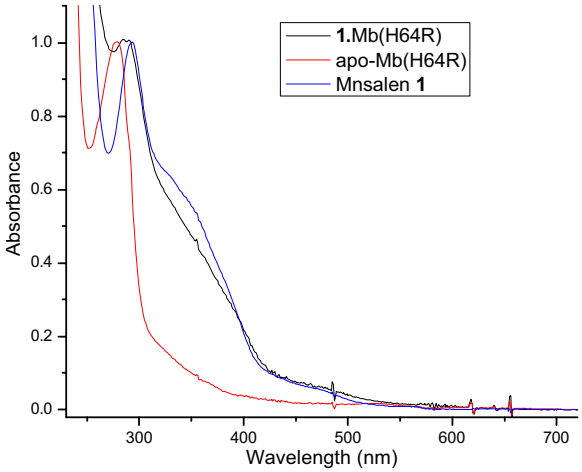
Figure S1. Electrospray mass spectrs of apo-Mb(H64X, X= H, F and R) and **1**·Mb(H64X, X= H, F and R)



1.Mb, Apo-Mb and Mnsalen



1.Mb(H64F), Apo-Mb(H64F), and Mnsalen



1.Mb(H64R), Apo-Mb(H64R), and Mnsalen

Figure S2. Optical spectra of **1**·Mb(H64X, X= H, F and R), Apo-Mb(H64X, X= H, F and R) and Mnsalen **1** in ammonia acetate buffer (50 mM, pH 5.1); **1**·Mb(H64X, X= H, F and R) (black line); apo-Mb(H64X, X= H, F and R) (red line); Mnsalen **1** (blue line).

Quantification of Catalyst Reactivity and Selectivity

Gas chromatography was performed on a Hewlett-Packard model 6890 equipped with a Hewlett-Packard 7683 auto injector using a 40 meter Astec Chiraldex G-TA capillary column.[6] Chromatographic conditions vary with analyte and are summarized in **Fehler! Verweisquelle konnte nicht gefunden werden.**.

Retention time for the over oxidation product sulfone was also determined. Injection size was 1μL. Determination of product chirality was accomplished by retention time comparison with independently prepared enantiomerically pure standards or by comparison with published retention times under similar conditions.[6]

Enantiomeric excess was determined by direct comparison of the corresponding peak areas in the chromatographic trace. Determination of the rate of reaction requires knowledge of the number of moles of product produced. However, it is not possible to directly measure these amounts in the reaction mixture both because of the low concentration of products produced and because water is detrimental to the GC column. To overcome this problem a known amount of internal standard was added to each reaction directly prior to extraction. Extraction efficiency of each analyte was calibrated relative to the internal standard by comparing peak areas from analogous extraction of buffer solutions with known concentration of internal standard and analytes.[7] That is, a plot of (analyte peak area/internal standard area) in the analyzed solution vs. (concentration of analyte/concentration of internal standard) in the pre-extracted solution was created. The slope and intercept of this plot were used to determine the concentration ratios in the pre-extraction solution. With this ratio and a known amount of internal standard added (and thus a known concentration) it is possible to determine the concentration (and from that the number of moles) of product. The turnover number was calculated as ((total moles of product)/ (moles of catalyst))/ (time in minutes).

Sulfoxidation Reaction Conditions

To 180 μL of 0.144 mM catalyst in buffer (the mixed buffer comprised to 50 mM each of sodium acetate, MES, MOPS, TRIS, adjusted to the certain pH.[8]) was added 10 μL 100 mM of the thioether substrate in MeOH. The resulting mixture was equilibrated for 30 min while stirring at 4ºC. The reaction was initiated by addition of 10 μL of 100

mM oxidant (H_2O_2 or PhIO) in buffer. Final reaction volume was 200 μL with final concentrations of 0.130 mM catalyst, 5 mM (39 equivalents) substrate and 5 mM (39 equivalents) oxidant. The reaction was allowed to proceed with stirring at 4ºC for 9.5 minutes at which point 10 μL of 100 mM internal standard in MeOH were added. After the designated reaction time (generally 10 minutes) the reaction was quenched by the addition of 250 μL of CH_2Cl_2. The reaction was quenched with addition of 10 μL of 1M NaS_2O_3 (a 10 fold excess of the oxidant added) and 250 μL of CH_2Cl_2. After vigorous mixing the entire solution (both aqueous and organic phases) was passed over a column of 50:50 Na_2SO_3: Na_2SO_4 to remove the water. The later method of quenching and sample work up was found to be more rapid. The resulting organic extracts were then evaporated just to dryness under gentle argon flow. The residue was dissolved in 50 μL of ethyl acetate that had been dried over molecular sieves. The solution was analyzed by GC as outlined in the chromatographic conditions tables.

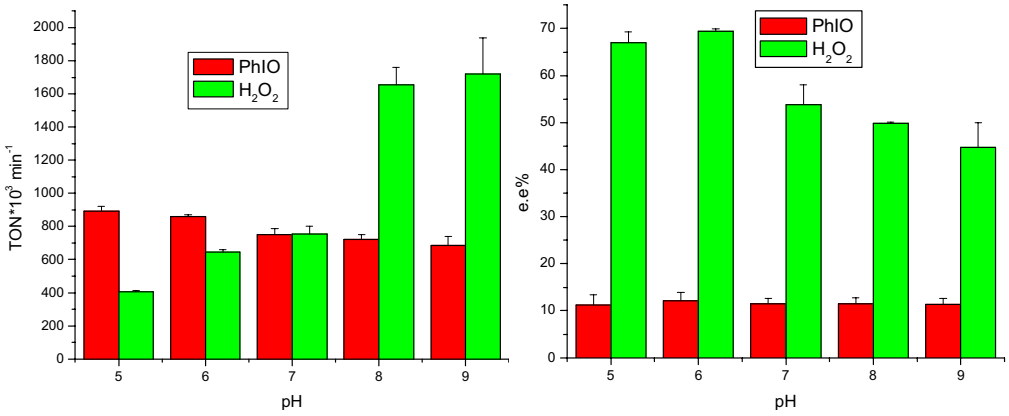
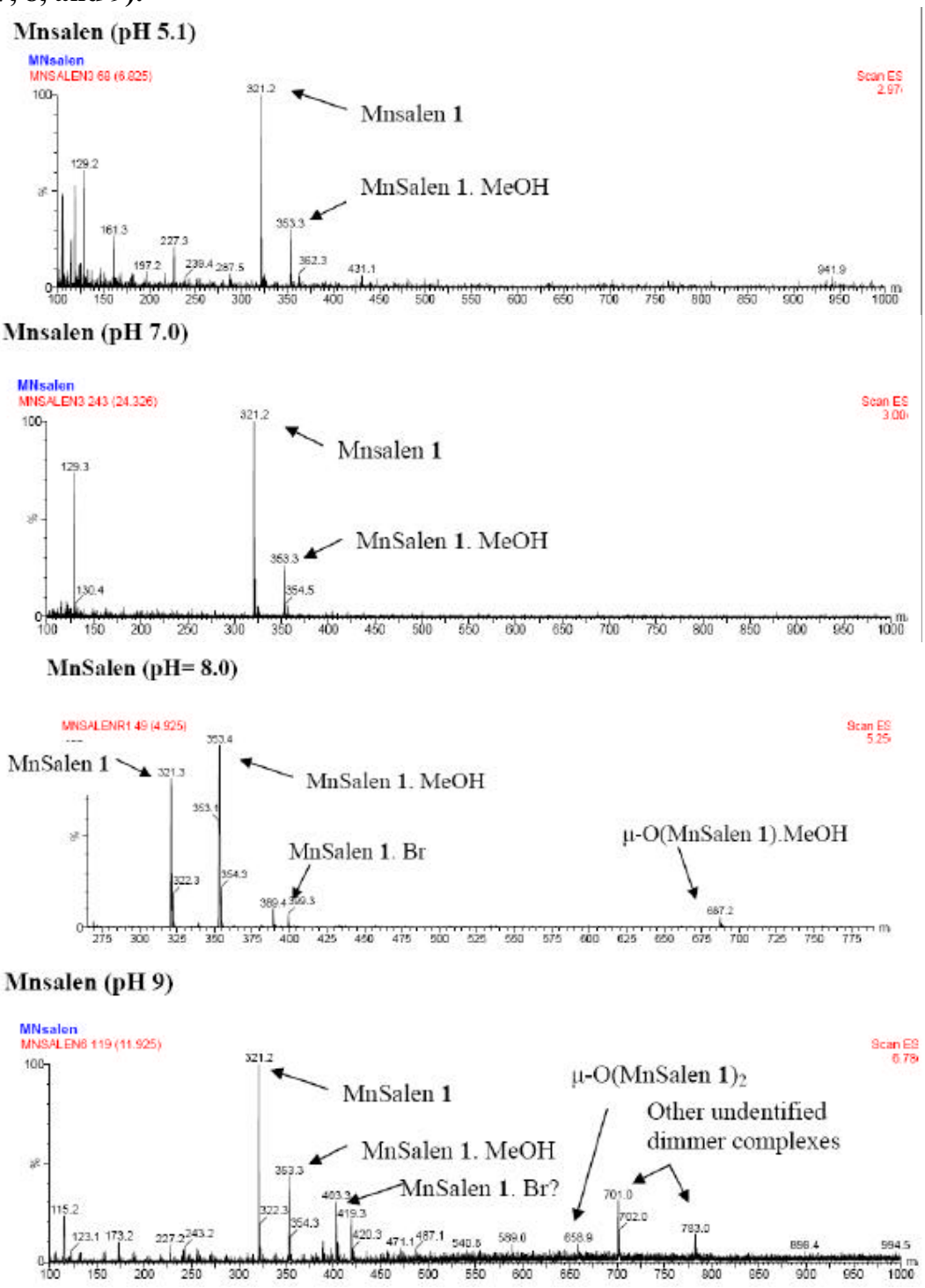


Figure S3. Oxidant effect on the sulfoxidation for **1•Mb** at different pH conditions (pH= 5.0, 6.0, 7.0, 8.0 and 9.0).

Figure S4. ESI Mass spectra of Mnsalen 1 in different pH conditions (5, 7, 8, and 9).



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