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Protein engineering to develop a redox insensitive endothelial nitric oxide synthase ☆

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ABSTRACT

The zinc tetrathiolate (ZnS_4) cluster is an important structural feature of endothelial nitric oxide synthase (eNOS). The cluster is located on the dimeric interface and four cysteine residues (C94 and C99 from two adjacent subunits) form a cluster with a Zn ion in the center of a tetrahedral configuration. Due to its high sensitivity to oxidants this cluster is responsible for eNOS dimer destabilization during periods of redox stress. In this work we utilized site directed mutagenesis to replace the redox sensitive cysteine residues in the ZnS_4 cluster with redox stable tetra-arginines. Our data indicate that this C94R/C99R eNOS mutant is active. In addition, this mutant protein is insensitive to dimer disruption and inhibition when challenged with hydrogen peroxide (H_2O_2). Further, the overexpression of the C94R/C99R mutant preserved the angiogenic response in endothelial cells challenged with H_2O_2. The over-expression of the C94R/C99R mutant preserved the ability of endothelial cells to migrate towards vascular endothelial growth factor (VEGF) and preserved the endothelial monolayer in a scratch wound assay. We propose that this dimer stable eNOS mutant could be utilized in the treatment of diseases in which there is eNOS dysfunction due to high levels of oxidative stress.

Introduction

Metallo-enzymes can coordinate zinc ions (Zn) through cysteine or histidine residues. The Zn ion can coordinate four ligands in a tetrahedral structure. In this work we focused on the ZnS_4 cluster in endothelial nitric oxide synthase (eNOS). Endothelial NOS, like all NOS isoforms, is a homodimeric enzyme with the ZnS_4 cluster at the dimeric interface. The cluster is formed by four sulfur atoms from two cysteine residues C94 and C99 from each monomer (Fig. 1). It is well established that the dimeric configuration is required for nitric oxide (NO) generation by NOS [1,2]. Thus, the ZnS_4 cluster is an important contributor to the proper folding of eNOS enzyme. However, the four sulfur atoms in the tetrahedral configuration are very sensitive to oxidation, leading to eNOS dimer disruption and attenuated NO production [3,4]. The distance between sulfur atoms in the ZnS_4 cluster is equal to the distance between sulfur atoms in (S–S) disulfide bond. Therefore, the formation of an intermediate with a two-center three-electron bond between two sulfur atoms is very favorable (Fig. 1) [5]. Further oxidation of the three-electron (S–S) intermediate requires significantly less energy than oxidation of free cysteine, therefore, oxidation of cysteine residues in the ZnS_4 cluster can occur even under conditions of mild oxidative stress.

As eNOS dependent vasodilation is an important mechanism regulating vascular tone, the disruption of eNOS activity under conditions of oxidative stress, can induce pathological changes in blood vessels that can lead to a number of diseases including atherosclerosis, diabetes mellitus and hypertension [6–8]. Thus, maintaining NO production is a primary goal in the treatment of cardiovascular disorders. The purpose of this study was to design an eNOS enzyme that is insensitive to oxidative stress. It has been previously reported that arginine rich structures can be stabilized by the formation of strong electrostatic interactions between arginine residues and negative ions such as phosphate or chloride [9]. We report here that the replacement of the ZnS_4 cluster with a tetra-arginine cluster results in a catalytically competent enzyme.

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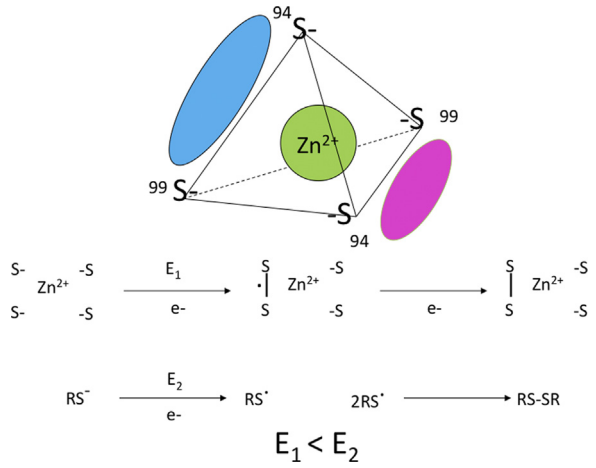

Fig. 1. The structure of Zn-tetrathiolate cluster. The Zn_4 cluster is composed of two cysteine residues (C94 and C99, human nomenclature) from each subunit of eNOS (cyan and magenta). Oxidation of cysteine residues within the Zn_4 cluster requires less energy due to formation of three electron S–S bond in the tetrahedral coordination.

This engineered eNOS is resistant to oxidative dimer disruption and is able to produce NO in an environment of enhanced oxidative stress.

Materials and methods

Molecular dynamic simulations

Molecular dynamic (MD) simulations were performed using Yasara. The Amber 99 all-hydrogen force field was used in the runs (22). For comparison, two 100 nsec simulations at 330 K were carried out using an eNOS dimeric structure. Simulations were carried out within a simulation cube filled with water molecules. All the atoms except those in the 70–125 aa region were fixed. The simulated systems contained ~2000 atoms. Snapshots were saved every 2 ns. Final structures after MD were structurally aligned using the Mustang algorithm in Yasara and the conformational changes were identified.

eNOS protein purification

For eNOS purification, 50 ml of terrific broth was premixed with ampicillin (100 mg/ml) and chlorophenicol (50 mg/ml), and inoculated with E. coli BL21 cells transformed with a polyHis-pCWeNOS plasmid containing wild type human eNOS sequence (9) or the mutant C94R/C99R. The polyHis-pCWeNOS vector was a gift from P. R. Ortiz de Montellano (University of California, San Francisco). The C94R/C99R eNOS mutant was prepared from the wildtype plasmid by Retrogen and sequenced to verify identity. Bacteria were grown overnight at 37 °C (260 rpm) then used to inoculate 2.8 L Fernabach flasks (6 × 1.5 L) again containing terrific broth (52 g/L) as the culture medium and supplemented with ampicillin (100 mg/ml), riboflavin (15 mg), and aminolevulinic acid (0.5 g). Flasks were placed on an orbital shaker and were allowed to grow at 37 °C (200 rpm). The OD_{600} was checked periodically during the growth period until it reached 0.8–1.0 (4–5 h) then adenosine-5′-triphosphate (ATP, 200 μM final concentration) and isopropyl-beta-D-thiogalactopyranoside, dioxane free (IPTG, 1 mM final concentration, to induce the T7 promoter) was added and the cells incubated for 18–20 h at 25 °C (200 rpm). Bacteria were then harvested by centrifugation using a FiberLite F6 6 × 1000 rotor at 4 °C (3500 rpm/2700g) for 20 min. The pellet was immediately transferred into lysis buffer (40 mM Tris–HCl, 5% glycerol, 1 mg/ml lysozyme, 100 mM NaCl, 4 mM FAD, 4 mM FMN, 100 μM BH_4, 5 mM L-arginine) and a protease inhibitor cocktail for use with histidine-tagged proteins (Sigma), ribonuclease A from bovine pancreas (Sigma), and deoxyribonuclease I from bovine pancreas (106 units, Sigma) was added. The pellet was gently rocked for 30 min at 4 °C, sonicated on ice, then subjected to ultracentrifugation at 4 °C (60,000 rpm/37,1000g) for 1 h and 45 min. The supernatant was loaded onto a Hisprep FF 16/10 column (charged with 0.1 M $NiSO_4$) using binding buffer (40 mM Tris–HCl, 100 mM NaCl, 5% glycerol, 30 mM imidazole, 100 μM BH_4, 100 μM L-arginine) at 0.1 ml/min flow. The column was washed with washing buffer (40 mM Tris–HCl, 300 mM NaCl, 5% glycerol, 30 mM imidazole, 100 μM BH_4, 100 μM L-arginine) using a flow rate of 1.5 ml/min, and a base line was obtained resulting in the washing out of non-histidine-tagged proteins. Elution of histidine-tagged protein was accomplished using elution buffer (40 mM Tris–HCl, 300 mM NaCl, 5% glycerol, 400 mM imidazole, 100 μM BH_4, 100 μM L-arginine) at 1.0 ml/min flow. Collected fractions were loaded for size-exclusion gel filtration on a HiLoad 26/60 Superdex 200 prep grade column using eNOS gel filtration buffer (60 mM Tris–HCl, 100 mM NaCl, 5% glycerol, 100 μM BH_4, 100 μM L-arginine) at 0.2 ml/min flow. Fractions were collected in 5 ml amounts for analysis by Coomassie blue staining and Western blot. Desalting was then performed for fractions containing eNOS using a HiPrep 26/10 desalting column and eNOS gel filtration buffer at flow rate of 0.5 ml/min. All purification steps were performed at 4 °C, and the purified protein was stored at −80 °C. Protein homogeneity was confirmed using Coomassie blue staining and Western blot with anti-eNOS antibody with 1:1000 dilutions (Transduction Labs). Final protein concentration was then measured in each fraction.

Gel filtration chromatography

To examine the extent of dimerization in the wildtype and mutant eNOS proteins we utilized analytical gel filtration. One hundred microlitres of each protein, at a concentration of 0.5 mg/ml, was injected into a Tosoh TSKgel G3000SW × l gel filtration column. Using a flow rate of 0.5 ml/min, monomer and dimer fractions were eluted in 100 mM phosphate buffer (pH = 7.0) using an HPLC system (GE) and analyzed by measuring the absorption at 260 nm.

Determination of NO_x levels

To measure NO production we utilized a chemiluminescence method. Wildtype eNOS and the C94R/C99R-eNOS were mixed with the cofactors calmodulin (10 μM) and BH_4 (40 μM) as well as the substrate L-arginine (100 μM) and Ca^{2+} (100 μM $CaCl_2$) in reaction buffer (50 mM HEPES, pH 7.4). The reaction was initiated with the addition of NADPH (10 μM). After 30 min of incubation at 37 °C the reaction mixture was analyzed for NO_x levels. In our experiments, potassium iodide (KI)/acetic acid reagent was prepared fresh daily by dissolving 0.05 g of KI in 7 ml of acetic acid. This reagent was added to a septum sealed purge vessel and bubbled with nitrogen gas. The gas stream was connected via a trap containing 1 N NaOH to a Sievers 280i Nitric Oxide Analyzer (GE). Samples were injected with a syringe through a silicone/Teflon septum. Results were analyzed by measuring the area under curve of the chemiluminescence signal using the Liquid software (GE). To carry out NO measurements in cells experiment, we utilized cell lysate and measured cellular NO content per mg of protein.

Measurement of superoxide levels

To detect superoxide generation, EPR measurements were performed using the spin trap, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine.HCl (CMH) as we have described [10,11].