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# Inactivation of cystathionine β-synthase with peroxynitrite

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ABSTRACT

Cystathionine β-synthase (CBS) is a homocysteine metabolizing enzyme that contains pyridoxal phosphate (PLP) and a six-coordinate heme cofactor of unknown function. CBS was inactivated by peroxynitrite, the product of nitric oxide and superoxide radicals. The $IC_{50}$ was ~150 μM for 5 μM ferric CBS. Stopped-flow kinetics and competition experiments showed a direct reaction with a second-order rate constant of $(2.4–5.0) \times 10^4\ M^{-1}\ s^{-1}$ (pH 7.4, 37 °C). The radicals derived from peroxynitrite, nitrogen dioxide and carbonate radical, also inactivated CBS. Exposure to peroxynitrite did not modify bound PLP but led to nitration of Trp208, Trp43 and Tyr223 and alterations in the heme environment including loss of thiolate coordination, conversion to high-spin and bleaching, with no detectable formation of oxoferryl compounds nor promotion of one-electron processes. This study demonstrates the susceptibility of CBS to reactive oxygen/nitrogen species, with potential relevance to hyperhomocysteinemia, a risk factor for cardiovascular diseases.

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## Introduction

Mammalian cystathionine β-synthase (CBS, E.C. 4.2.1.22)[1] catalyzes the condensation of homocysteine with serine to form cystathionine and water in the first step of the transsulfuration pathway that yields cysteine. CBS can also catalyze the formation of hydrogen sulfide, a novel gasotransmitter with signaling and cytoprotective effects [1].

Homocysteine is a toxic metabolite that is elevated in hyperhomocysteinemic patients and constitutes an independent risk factor for cardiovascular diseases, including atherosclerosis. Additionally, elevations in plasma homocysteine correlate with other pathologies such as neurological disorders [2–4]. The most common cause of inherited hyperhomocysteinemia is deficiency in CBS. Presently, 140 mutations have been described and are listed at http://cbs.lf1.cuni.cz/index.php.

The human full-length CBS is a homotetramer of 63 kDa subunits that exhibit a modular organization [5,6]. Each subunit contains at the N-terminal end the binding site for a heme b-type cofactor. The middle catalytic core has a pyridoxal 5′-phosphate (PLP) cofactor essential for catalysis and a CXXC disulfide oxidoreductase motif whose function has not been determined. Finally, the C-terminal region contains a tandem repeat of two "CBS domains" that probably bind S-adenosyl-L-methionine (SAM), an allosteric activator [7]. The regulatory region can be cleaved proteolytically by trypsin, yielding a truncated dimeric enzyme with 45 kDa subunits that is more active, stable, and unresponsive to SAM [5]. This truncated form can be observed in liver cells exposed to the proinflammatory cytokine, tumoral necrosis factor [8].

CBS is the only known PLP-dependent enzyme that also contains heme [9]. The heme cofactor is six-coordinate and in the low-spin state with cysteine (Cys52, human numbering) and histidine (His65) as axial ligands [10–12]. The UV–visible absorption spectrum of ferric CBS (Fe(III)CBS) shows a $\delta$ peak at 364 nm, a Soret peak at 428 nm and a broad absorption feature at 550 nm corresponding to the αβ bands [13]. The coordination between the thiolate and ferric iron can be observed at high enzyme

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[1] *Abbreviations used:* CBS, cystathionine β-synthase; SAM, S-adenosyl-L-methionine; PLP, pyridoxal 5′-phosphate; DTPA, diethylenetriaminepentaacetic acid; ROA, reverse order of addition; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); LMCT, ligand to metal charge transfer band; EPR, electron paramagnetic resonance or electron spin resonance; p-HPA, p-hydroxyphenylacetic acid; HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; MS, mass spectrometry; $IC_{50}$, concentration of peroxynitrite needed to inactivate by 50%.

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concentrations (~100 μM) in the low energy region by the presence of two bands at 645 and 705 nm [14]. Upon reduction to Fe(II)CBS with sodium dithionite or titanium citrate, the Soret peak shifts to 449 nm and the αβ bands are resolved into two peaks at 571 and 540 nm [13].

The function of the heme is yet unknown. The six-coordinated hemethiolate has very low reactivity in the ferric state and this stability is reflected by the fairly low reduction potential of −0.291 V for the truncated enzyme [15]. It is unreactive towards typical ferric heme ligands like cyanide, fluoride, azide, pyridines, amines, isonitriles and imidazoles [16,17]. However, treatment of the ferric enzyme with high concentrations of the thiol chelator mercuric chloride ($HgCl_2$) results in the conversion of the six-coordinate low-spin heme to a five-coordinate high-spin species, which is paralleled by a loss of activity [12,18]. Indeed, perturbations in the heme environment lead to enzyme inactivation. For example, reduction at high temperatures, which leads to loss of the native cysteinate ligand and formation of a ferrous species with a neutral ligand that absorbs at 424 nm, inactivates the enzyme [19]. Analogously, mutation of the axial ligands His65 and Cys52 decrease activity despite the relatively high PLP content of the resulting protein [20]. It has been postulated that communication between the PLP and heme domains, distant 20 Å, may be mediated by an α-helix (helix 8) that interacts at one end with the Cys52 ligand via Arg266, and at the other end with PLP [21,22].

Peroxynitrite[2] ($ONOO^-$) is a powerful oxidizing and nitrating agent that is produced in biological systems by the reaction of superoxide ($O_2^{\cdot -}$) and nitric oxide ($^{\cdot}NO$) [23,24]. Due to its $pK_A$ of 6.8, the anion predominates (80% at pH 7.4) under physiological conditions. The conjugated acid homolyzes at a rate of 0.9 $s^{-1}$ (37 °C, pH 7.4), giving free hydroxyl ($^{\cdot}OH$) and nitrogen dioxide ($^{\cdot}NO_2$) radicals in 30% yield, which can nitrate aromatic residues such as tyrosine and tryptophan. The preferential biotargets of peroxynitrite are metal centers, carbon dioxide, and sulfur and selenium compounds (for recent reviews see [25,26]). The reaction with carbon dioxide ($4.6 \times 10^4\ M^{-1}\ s^{-1}$ at 37 °C and pH 7.4) [27] leads to the formation of carbonate radical ($CO_3^{\cdot -}$) and nitrogen dioxide in 35% yield.

Peroxynitrite reacts with ferric heme proteins including hemethiolates at rates ranging from $10^4$ to $10^7\ M^{-1}\ s^{-1}$. The reaction usually proceeds through the intermediate formation of oxo-ferryl species [28–30]. Depending on the protein, the outcome can be decomposition of peroxynitrite to nitrate and nitrite or enhancement of one-electron oxidative processes, sometimes leading to enzyme inactivation and increased nitration of exogenous or endogenous aromatic residues [29,31,32].

Compelling evidence shows that reactive oxygen and nitrogen species are central mediators of several pathological conditions including cardiovascular disease. The increased formation of peroxynitrite in vivo is confirmed by the observed effect of superoxide radical on nitric oxide bioavailability and by the detection of nitrotyrosine (for reviews see [33,34]). Since peroxynitrite interaction with CBS could lead to enzyme inactivation and homocysteine accumulation, we investigated the reactivity of peroxynitrite with the human truncated Fe(III)CBS dimer in this study.

## Materials and methods

### *Peroxynitrite synthesis*

The stock solutions were prepared from acidified hydrogen peroxide and sodium nitrite in a tandem quenched-flow mixing apparatus [35]. The solutions were treated with manganese dioxide to remove residual hydrogen peroxide. The concentration of peroxynitrite was determined from its absorbance at 302 nm ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$ [36]). The concentration of nitrite present as contaminant was measured with the Griess reagent [37] after decay of peroxynitrite to nitrate in monobasic sodium phosphate solution. Nitrite content was always less than 30% of peroxynitrite. Peroxynitrite was diluted in 0.1 M NaOH immediately before use. Stock solutions were stored at −80 °C, used only once after thawing, and then discarded.

### *Enzyme purification*

Truncated human CBS lacking 143 amino acids at the C-terminus (CBSΔC143) was purified as a fusion protein with glutathione S-transferase using the *Escherichia coli* expression vector pGEXCBSN [38], kindly provided by Dr. Warren Kruger, Fox Chase Cancer Center, Philadelphia. The protein was purified as described previously [13] through affinity chromatography with glutathione sepharose and anion exchange chromatography. The glutathione S-transferase tag was cleaved by limited proteolysis using thrombin. The protein obtained is an active and stable dimer of 45 kDa subunits that does not bind SAM.

### *Enzymatic assays*

CBS activity in Tris buffer (0.1 M, pH 8.3) was determined using the ninhydrin assay [39]. The specific activity of the enzyme was ~590 $\mu mol\ h^{-1}\ mg^{-1}$ at 37 °C (~9.8 $\mu mol\ min^{-1}\ mg^{-1}$), similar to previously reported [5]. The protein concentration was determined by the Bradford method using albumin as a standard. The ratio of absorbance at 280 and 428 nm was 1.1. Thiols were measured spectrophotometrically using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, $\varepsilon_{412} = 13{,}600\ M^{-1}\ cm^{-1}$ [40]), after ultrafiltration with Ultrafree 0.5 centrifugal filter devices (Millipore) to remove interference from the heme and PLP at 412 nm.

### *Exposure of CBS to peroxynitrite*

Peroxynitrite was added to the enzyme in sodium phosphate buffer of the specified concentration and pH, in the presence of diethylenetriaminepentaacetic acid (DTPA, 0.1 mM) to eliminate potential metal trace interference. In order to minimize changes in pH due to NaOH present in peroxynitrite solutions, additions were always <5% of total volume. The enzyme was incubated for 2 min at 37 °C before and after peroxynitrite was added. Reverse order of addition (ROA) controls, where peroxynitrite was decomposed in buffer before mixing with enzyme, were performed with the higher concentration of peroxynitrite used in each experiment. Phosphate buffers were prepared daily avoiding the use of NaOH in order to minimize bicarbonate/carbon dioxide contamination. For experiments in which sodium bicarbonate (25 mM) was specifically added, the initial pH of the buffer was lowered so that the final value was 7.4 ± 0.1. The buffers were prepared immediately before the experiment and used within 10 min to minimize diffusion of carbon dioxide out of the solution.

### *CBS reduction*

Ferrous CBS was obtained by the addition of known amounts of sodium dithionite ($Na_2S_2O_4$). Dithionite stock solutions were prepared in degassed 0.1 N NaOH and quantified by ferricyanide reduction ($\varepsilon_{420} = 1020\ M^{-1}\ cm^{-1}$) [41] assuming a 2:1 stoichiometry.

[2] The term peroxynitrite is used to refer to both peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH). IUPAC-recommended names are oxoperoxonitrate(1-) and hydrogen oxoperoxonitrate, respectively.

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