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Kinetics of Reversible Reductive Carbonylation of Heme in Human Cystathionine β-Synthase

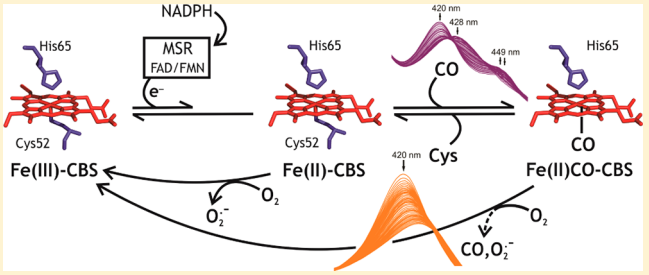
Sebastián Carballal,[†,‡,§] Ernesto Cuevasanta,[†,§] Inés Marmisolle,[†,§] Omer Kabil,[∥] Carmen Gherasim,[∥] David P. Ballou,[∥] Ruma Banerjee,*[,∥] and Beatriz Alvarez*[,†,§]

[†]Laboratorio de Enzimología, Facultad de Ciencias, [‡]Departamento de Bioquímica, and [§]Center for Free Radical and Biomedical Research, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay

[∥]Department of Biological Chemistry, Medical Center, University of Michigan, Ann Arbor, Michigan 48109-0600, United States

Supporting Information

ABSTRACT: Cystathionine β-synthase (CBS) catalyzes the condensation of homocysteine with serine or cysteine to form cystathionine and water or hydrogen sulfide (H_2S), respectively. In addition to pyridoxal phosphate, human CBS has a heme cofactor with cysteine and histidine as ligands. While Fe(III)-CBS is inert to exogenous ligands, Fe(II)-CBS can be reversibly inhibited by carbon monoxide (CO) and reoxidized by O_2 to yield superoxide radical. In this study, we have examined the kinetics of Fe(II)CO-CBS formation and reoxidation. Reduction of Fe(III)-CBS by dithionite showed a square root dependence on concentration, indicating that the reductant species was the sulfur dioxide radical anion ($SO_2^{\bullet-}$) that exists in rapid equilibrium with $S_2O_4^{2-}$. Formation of Fe(II)CO-CBS from Fe(II)-CBS and 1 mM CO occurred with a rate constant of $(3.1 \pm 0.4) \times 10^{-3}$ s^{-1} (pH 7.4, 25 °C). The reaction of Fe(III)-CBS with the reduced form of the flavoprotein methionine synthase reductase in the presence of CO and NADPH resulted in its reduction and carbonylation to form Fe(II)CO-CBS. Fe(II)-CBS was formed as an intermediate with a rate constant of $(9.3 \pm 2.5) \times 10^2$ M^{-1} s^{-1}. Reoxidation of Fe(II)CO-CBS by O_2 was multiphasic. The major phase showed a hyperbolic dependence on O_2 concentration. Although H_2S is a product of the CBS reaction and a potential heme ligand, we did not find evidence of an effect of exogenous H_2S on activity or heme binding. Reversible reduction of CBS by a physiologically relevant oxidoreductase is consistent with a regulatory role for the heme and could constitute a mechanism for cross talk among the CO, H_2S, and superoxide signaling pathways.



Cystathionine β-synthase (CBS) is a pyridoxal phosphate (PLP)-dependent enzyme that plays a central role in mammalian sulfur metabolism. It catalyzes the condensation of serine with homocysteine to give cystathionine and water in the first step of the canonical transsulfuration pathway that leads to cysteine, the limiting substrate for glutathione synthesis. CBS also catalyzes the condensation of homocysteine and cysteine to form cystathionine and hydrogen sulfide (H_2S), which modulates several physiological functions in the cardiovascular, gastrointestinal, and nervous systems.[1−3] Mutations in CBS are the single most common cause of severe hyperhomocysteinemia.[4] Increased levels of homocysteine in plasma are associated with a range of pathologies, including cardiovascular diseases and neural tube defects.[5,6]

In addition to PLP, human CBS harbors a *b*-type heme cofactor that is low-spin and six-coordinate in both the ferric and ferrous states.[7] The axial heme ligands in human CBS are cysteine (Cys52) and histidine (His65).[8−11] The UV−visible absorption spectrum of Fe(III)-CBS shows a Soret peak at 428 nm and broad α/β bands centered at 550 nm. Upon reduction to Fe(II)-CBS, the Soret peak shifts to 449 nm and the α/β bands are resolved into two peaks at 571 and 540 nm, respectively.[12]

The function of the heme in CBS remains unknown. A direct catalytic role in the PLP-mediated enzymatic mechanism was initially excluded by spectroscopic studies[13] and is consistent with its absence from the highly homologous yeast and *Trypanosoma cruzi* enzymes, which catalyze the same overall reaction.[14,15] Although crystal structures reveal a considerable distance (~20 Å) between the heme and PLP cofactors,[10,11,16] a regulatory role has been suggested on the basis of the observation that perturbations in the heme coordination affect the equilibrium between PLP tautomers and modulate CBS activity.[17] Bidirectional communication between the heme and PLP sites occurs through an α-helix that interacts at one end with the Cys52 heme ligand via Arg266 and at the other end with PLP via two conserved threonine residues (Thr257 and Thr260).[17−22] Ferric CBS is relatively stable, as reflected by the fairly low reduction potential of −0.350 V,[20] and is inert to typical ferric heme ligands.[23,24] It is sensitive only to the thiol chelator, mercuric chloride, and to the strong oxidant, peroxynitrite.[25,26] In contrast, ferrous heme can bind ligands

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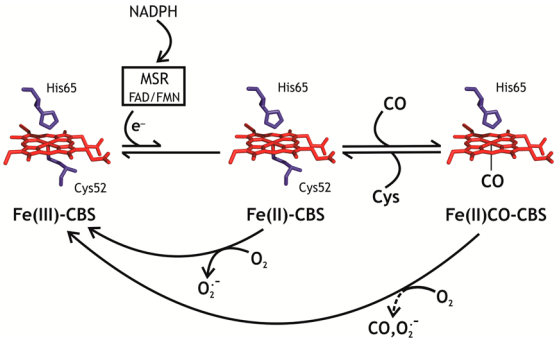
such as isonitriles, cyanide, nitric oxide ($^{\bullet}$NO), and carbon monoxide (CO), although with different affinities.[23,24,27] CO binds to ferrous CBS by displacing the axial cysteine ligand and forming Fe(II)CO-CBS (Scheme 1), a species with a Soret

Scheme 1. Reversible Reduction of CBS by MSR in the Presence of CO



maximum at 420 nm and decreased catalytic activity. The K_i value for CO is 5.6 μM, which might be in a physiologically relevant range for endogenous CO concentrations generated by heme oxygenases in different tissues.[23,28,29] In addition, Fe(II)-CBS can slowly decay to an inactive form that absorbs at 424 nm [Fe(II)424-CBS], in which the cysteine is replaced by a neutral ligand of unknown identity.[30] Finally, the reaction of Fe(II)-CBS with O_2 occurs rapidly with a second-order rate constant of (1.13 ± 0.05) × 10^5 M^{-1} s^{-1} (pH 7.4, 25 °C), leading to formation of Fe(III)-CBS (which is fully active) and superoxide radical anion ($O_2^{\bullet-}$). No intermediates are detected during the oxidation reaction, which exhibits a linear dependence on O_2 concentration, suggesting that it occurs via an outer sphere electron transfer.[31]

Knowing whether the heme can be reduced by biological systems is central to evaluating its possible roles in sensing CO or producing $O_2^{\bullet-}$ in the cellular milieu. The low heme reduction potential in CBS makes it a challenging reduction. On the other hand, the heme appears to be fairly surface-exposed in the structures of both truncated and full-length CBS, indicating that it should be accessible to cellular reducing systems.[10,11,16] We have recently demonstrated that human methionine synthase reductase (MSR) and NADPH can reduce CBS (Scheme 1). In the presence of CO, reduction is coupled to carbonylation, leading to formation of Fe(II)CO-CBS with concomitant loss of enzyme activity.[32] MSR is a cytosolic flavoprotein oxidoreductase that harbors both FAD and FMN.[33] The reduction potentials for the semiquinone/hydroquinone couples are −0.291 and −0.227 V for FAD and FMN, respectively.[34] MSR has been shown to mediate the transfer of an electron from NADPH to the cob(II)alamin cofactor of methionine synthase. The cob(II)alamin/cob(I)-alamin reduction potential in methionine synthase is estimated to be −0.526 V,[35] and kinetic coupling of reduction with methylation by *S*-adenosylmethionine results in reductive methylation and reactivation of methionine synthase.[33,34]

In this study, we have investigated the kinetics of the reduction of the heme in CBS by dithionite and MSR/NADPH in the presence of CO. We have also examined the kinetics of Fe(II)CO-CBS reoxidation by O_2 and assessed the effect of H_2S on CBS activity. Our results provide insights into the kinetics of heme-mediated regulation of human CBS.

EXPERIMENTAL PROCEDURES

Enzyme Purification and Assays. Recombinant full-length human CBS, the truncated ΔC143 variant, and MSR were purified as described previously.[12,33,36] CBS activity was measured using either the ninhydrin method[37] or the radiolabel assay.[12] Specific activities of the CBS preparations used in this study were 320 ± 26 and 878 ± 52 μmol h^{-1} mg^{-1} for full-length CBS and the truncated variant, respectively. Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard.[38] The absorption spectrum of MSR exhibited maxima at 380 and 454 nm and a shoulder at 480 nm. The A_{280}/A_{454} ratio was ∼5.5, similar to that reported previously.[36] Reduction of MSR by NADPH under aerobic conditions led to a decrease in absorbance at 454 nm and the appearance of a small shoulder in the vicinity of 585 and 640 nm, consistent with the formation of an air-stable semiquinone. The specific activity of MSR, determined by NADPH-dependent cytochrome *c* reduction, was 3.8 ± 0.2 μmol min^{-1} mg^{-1} at 37 °C, similar to that reported previously.[36]

Kinetics of Reduction of Fe(III)-CBS by Dithionite. Sodium dithionite stock solutions were prepared in degassed 0.1 M NaOH and quantified by ferricyanide reduction (ε_{420} = 1020 M^{-1} cm^{-1}) assuming a 2:1 (ferricyanide:dithionite) stoichiometry.[39,40] An anaerobic solution of Fe(III)-CBS (2 μM) was mixed with dithionite (0.1−4 mM) in phosphate buffer (0.2 M, pH 7.4, 0.2 mM DTPA) at 25 °C. Formation of Fe(II)-CBS was followed at 449 nm in a Varian Cary 50 spectrophotometer coupled to an Applied Photophysics RX2000 stopped-flow machine. No changes in pH were detected after mixing.

Kinetics of Reaction of Fe(II)-CBS with CO. A solution of Fe(III)-CBS in CO-saturated phosphate buffer (0.1 M, pH 7.4) was mixed with a CO-saturated water solution to which dithionite had been added at low concentrations, using a stopped-flow spectrophotometer (SX.MV18, Applied Photophysics) with a diode array detector, placed inside an anaerobic chamber (Vacuum Atmospheres Co., Hawthorne, CA) that had a 95:5 N_2/H_2 atmosphere and <1 ppm O_2.

Kinetics of Reduction of Fe(III)-CBS by MSR and NADPH in the Presence of CO. Quartz cuvettes containing CBS, MSR, and NADPH were prepared in the anaerobic chamber and sealed with a rubber septum, and their headspace was purged with CO for 15 min outside the anaerobic chamber. Then, UV−visible absorption spectra were recorded every minute up to ∼50 min. The ionic strength dependence of the reduction of CBS by MSR was determined in 25, 50, or 100 mM potassium phosphate buffer (pH 7.4) to which variable amounts of KCl had been added to obtain final ionic strengths ranging from 75 to 760 mM. The ionic strength (μ) was calculated according to the equation $\mu = {}^1/_2\sum(M_iZ_i^2)$, where M_i and Z_i are the molarity and net charge of the ion, respectively. Rapid reaction kinetic studies were performed using the stopped-flow spectrophotometer placed inside the anaerobic chamber or a Hi-Tech Scientific SF-61DX stopped-flow spectrophotometer in the photodiode array mode placed outside the anaerobic chamber. In the latter case, to minimize O_2 contamination, the stopped-flow instrument was flushed with an anaerobic solution of protocatechuate (200 μM) and protocatechuate 3,4-dioxygenase (∼1 μM) in phosphate buffer (0.1 M, pH 7.4).[41] This solution was replaced with O_2-free buffer before introduction of the anaerobic reaction mixtures.

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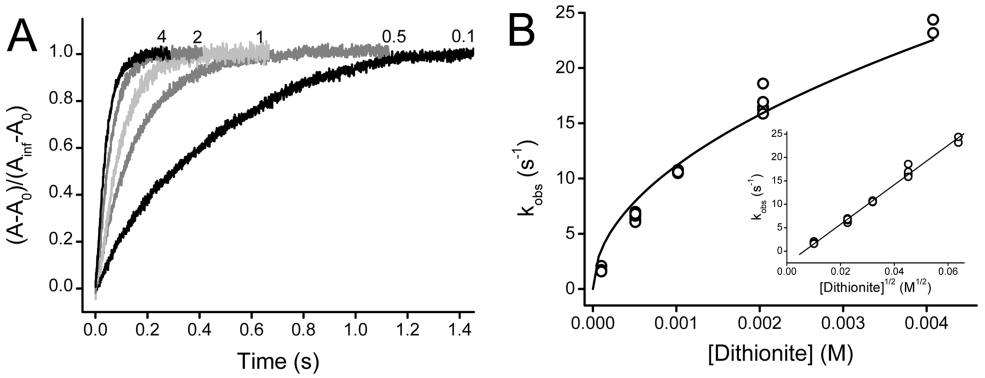

Figure 1. Kinetics of reduction of Fe(III)-CBS by dithionite. (A) Fe(III)-CBS (2 μM) was mixed with sodium dithionite (0.1, 0.5, 1, 2, and 4 mM, gray traces) in sodium phosphate buffer (0.2 M, pH 7.4, 0.2 mM DTPA) under anaerobic conditions at 25 °C. All concentrations given are after mixing. The absorbance at 449 nm was fit to a single-exponential function to determine the k_{obs}. (B) The observed rate constants (k_{obs}) were plotted vs dithionite concentration and the square root of the dithionite concentration (inset).

CBS and MSR solutions were made anaerobic in glass tonometers by repeated cycles of vacuum and equilibration with argon and left under a positive pressure of argon. Anaerobic 1 μM CO-saturated Fe(III)-CBS was mixed with increasing concentrations (2, 5, or 10 μM) of MSR prereduced with 1 mM NADPH, and spectra were recorded from 0.02 to 1425 s. The experiments were performed at 25.0 ± 0.1 °C.

Kinetics of Reoxidation of Fe(II)CO-CBS by O_2. Fe(II)CO-CBS was obtained by first placing anaerobic Fe(III)-CBS (10 μM) in phosphate buffer (0.2 M, pH 7.4) in a tonometer equipped with an L-shaped side arm fused to a spectrophotometer quartz cuvette. Next, CBS was stoichiometrically reduced with dithionite delivered via a gastight Hamilton syringe, monitoring the heme redox status spectrophotometrically by tipping the solution from the tonometer to the cuvette. Because the $O_2^{\bullet-}$ radical can be formed in the presence of O_2, care was taken to avoid high concentrations of dithionite, and 0.25 μM superoxide dismutase (SOD) was included. Following reduction, CBS was saturated with CO by consecutive cycles of vacuum and CO flow. Fe(II)CO-CBS formation was confirmed by the appearance of the 420 nm peak. The resulting Fe(II)CO-CBS solution was then mixed with solutions containing a series of O_2 concentrations obtained by equilibrating distilled water for 20 min with air (21% O_2) or with certified O_2/N_2 gas mixtures (10, 50, and 100% O_2) at 25.0 ± 0.1 °C.

Exposure of CBS to H_2S. Solutions of H_2S were freshly prepared by dissolving NaHS in ultrapure water and kept in sealed vials. The concentration of stocks was standardized by iodometric titration.[42] The final solutions contained a mix of H_2S and HS^-, governed by the pK_{a1} value of 7.0.[43] At pH 7.4, sulfide is distributed into 72% HS^- and 28% H_2S. In some experiments, dithionite (280 μM) was added to the NaHS stock solution (0.13 M). When required, reduction of Fe(III)-CBS was achieved by addition of dithionite (60 μM) in nitrogen-purged sealed vials.

Simulations and Data Analysis. Computer-assisted kinetic simulations were performed with GEPASI.[44] Kinetic traces from the stopped-flow experiments were analyzed and fit using Pro-Data Viewer (Applied Photophysics), KinetAsyst3 (Hi-Tech Scientific, Salisbury, U.K.), or Origin version 6.1. All experiments were repeated at least twice, and representative data are shown.

RESULTS

Kinetics of Reduction of Fe(III)-CBS by Dithionite. When Fe(III)-CBS was mixed with excess dithionite, the heme was reduced rapidly. The formation of Fe(II)-CBS followed exponential kinetics, in agreement with the reaction being first-order in Fe(III)-CBS (Figure 1A). The observed rate constant (k_{obs}) did not increase linearly with the concentration of dithionite. Instead, as with other heme proteins,[45−47] the k_{obs} showed a dependence on the square root of the dithionite concentration (Figure 1B) and plots of k_{obs} versus $[\text{dithionite}]^{1/2}$ were linear (Figure 1B, inset). These results are consistent with the reductant being the sulfur dioxide radical anion ($SO_2^{\bullet-}$), formed by rapid homolysis of dithionite as described by eqs 1 and 2.

$$S_2O_4^{2-} \rightleftharpoons 2SO_2^{\bullet-} \quad (1)$$

$$\text{Fe(III)-CBS} + SO_2^{\bullet-} \rightarrow \text{Fe(II)-CBS} + SO_2 \quad (2)$$

The forward and reverse rate constants for eq 1 are as follows: $k_1 = 2.5\ s^{-1}$, and $k_{-1} = 1.8 \times 10^9\ M^{-1}\ s^{-1}$ (25 °C).[45,48] This mechanism yields k_{obs} as described by eq 3, where k_2 is the second-order rate constant for reduction of Fe(III)-CBS by $SO_2^{\bullet-}$

$$k_{obs} = k_2(k_1/k_{-1})^{1/2}[S_2O_4^{2-}]^{1/2} \quad (3)$$

From the fit of the values of k_{obs} to eq 3 (Figure 1B), the dependence on $[S_2O_4^{2-}]^{1/2}$ was found to be $k_2(k_1/k_{-1})^{1/2} = 353 \pm 7\ M^{-1/2}\ s^{-1}$ and k_2 was calculated to be $(9.5 \pm 0.2) \times 10^6\ M^{-1}\ s^{-1}$ (pH 7.4, 25 °C).

Kinetics of Reaction of Fe(II)-CBS with CO. Fe(II)CO-CBS formation was first analyzed by mixing anaerobic Fe(III)-CBS (1 μM) with dithionite (26 μM) in a stopped-flow spectrophotometer, in the presence of CO (1 mM). Complete heme reduction was achieved in <10 s, which is consistent with the fast kinetics of CBS reduction by dithionite described above. A half-time of 0.4 s for heme reduction can be estimated under these conditions. Thus, the first spectrum shown is of the reduced CBS heme. Once reduced, Fe(II)-CBS reacted with CO and the heme Soret band shifted from 449 nm to a new maximum at 420 nm, consistent with previously reported spectra (Figure 2).[23] The kinetics of binding of CO to Fe(II)-CBS, monitored by the decrease in absorbance at 449 nm for 500 s, were fit to a single-exponential function (Figure 2, inset) and yielded a value for k_{obs} of $(3.1 \pm 0.4) \times 10^{-3}\ s^{-1}$ at 1 mM

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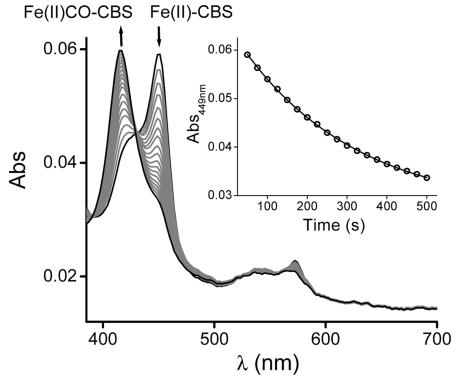

Figure 2. Kinetics of reaction of Fe(II)-CBS with CO. Stopped-flow UV−visible absorption spectra after mixing Fe(III)-CBS in CO-saturated phosphate buffer (0.1 M, pH 7.4) with dithionite in CO-saturated water at 25 °C. The concentrations of Fe(III)-CBS, dithionite, and CO after mixing were 0.5 μM, ∼26 μM, and ∼1 mM, respectively. Spectra were collected every 25 s for 500 s. The arrows indicate the direction of the absorbance change over time. The inset shows the absorbance at 449 nm (○) plotted vs time. The solid line represents the fit to an exponential function.

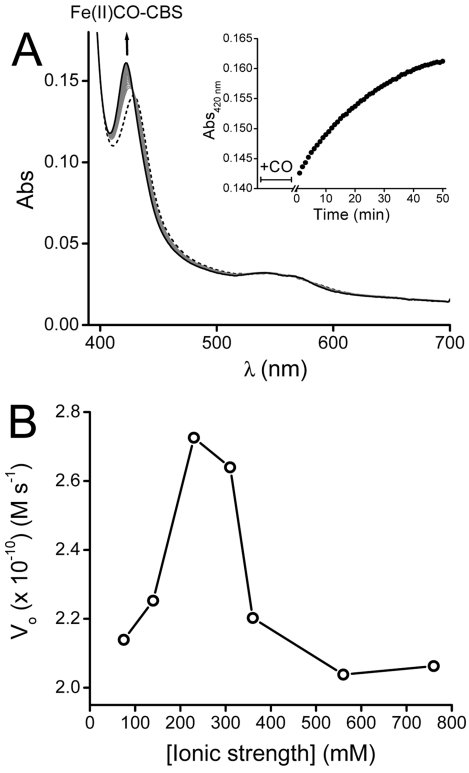

Figure 3. Reduction of CBS by MSR in the presence of CO. (A) Fe(III)-CBS (1 μM) was mixed with MSR (2 μM) and NADPH (1 mM) in phosphate buffer (0.1 M, pH 7.4) at 25 °C under anaerobic conditions. Then, the reaction mixture was saturated with CO (∼1 mM) for 15 min, and changes in the UV−visible spectrum were monitored. The dotted line represents the spectra of the reaction mixture before saturation with CO. The inset shows the absorbance at 420 nm plotted vs time. (B) Ionic strength dependence of the reductive carbonylation of CBS by MSR. The reaction mixtures were prepared in phosphate buffer with increasing KCl concentrations as described in Experimental Procedures. The rates of the reaction (v_o) were determined from the linear increment of absorbance at 420 nm in the first 10 min.

CO (pH 7.4, 25 °C). After 500 s, the absorbance maximum shifted slowly to 414 nm. The origin of this spectral shift is presently not understood and was not studied further. Of note, the carbonylated species obtained for *Drosophila melanogaster* CBS has a peak maximum at 414 nm.[49]

Kinetics of Reduction of Fe(III)-CBS by MSR and NADPH in the Presence of CO. In the absence of CO (Figure S1 of the Supporting Information), no formation of Fe(II)-CBS was detected, even at MSR:CBS ratios of 1:1, 2:1, or 4:1. In the presence of CO, conversion to Fe(II)CO-CBS with a Soret maximum near 420 nm was observed (Figure 3A). This suggests that the heme in CBS is first reduced by MSR in the presence of NADPH and then reacts with CO forming Fe(II)CO-CBS. The apparent rate for Fe(II)CO-CBS formation was estimated to be $\sim 2 \times 10^{-10}$ M s^{-1} during the first minutes of spectral registration (0.1 M phosphate buffer, pH 7.4, ionic strength of ∼260 mM). Dithionite was used to obtain the difference extinction coefficient between Fe(II)CO-CBS and Fe(III)-CBS at 420 nm [57360 M^{-1} cm^{-1} (Figure S2 of the Supporting Information)]. When the rate was divided by the concentrations of MSR and CBS, an apparent second-order rate constant on the order of 10^2 M^{-1} s^{-1} was estimated for Fe(II)CO-CBS formation. Exponential fits of the time courses yielded similar estimations (not shown). Next, the dependence of the apparent rate of Fe(II)CO-CBS formation on ionic strength was examined and found to be bell-shaped, with maximal rates observed at ∼200 mM (Figure 3B).

Because the initial reaction time course was not captured in the experiments described above, stopped-flow experiments were performed. As shown in Figure 4, when anaerobic CO-saturated Fe(III)-CBS was mixed with NADPH-reduced MSR, Fe(II)-CBS was formed in <200 s, followed by a time-dependent formation of Fe(II)CO-CBS as described by eqs 4 and 5.

$$\text{Fe(III)-CBS} + \text{MSR}_{red} \rightleftharpoons \text{Fe(II)-CBS} + \text{MSR}_{ox} \quad (4)$$

$$\text{Fe(II)-CBS} + \text{CO} \rightarrow \text{Fe(II)CO-CBS} \quad (5)$$

To determine the rate constant (k_4) for reduction of the heme by MSR to form Fe(II)-CBS, the absorbance traces at

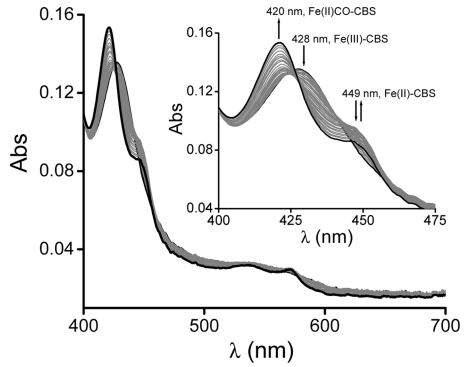

Figure 4. Stopped-flow kinetics of reduction of CBS by MSR in the presence of CO. A solution containing Fe(III)-CBS (1 μM) and CO (∼500 μM) was mixed with MSR (2 μM) prereduced by NADPH (500 μM) in phosphate buffer (0.1 M, pH 7.4 with 0.1 mM DTPA) at 25 °C under anaerobic conditions (concentrations after mixing). Spectra were collected from 0.75 to 1425 s. For the sake of clarity, only selected spectra are shown. The inset shows an expanded view of the UV−visible spectra between 400 and 475 nm. The arrows indicate the direction of the spectral evolution as a function of time.

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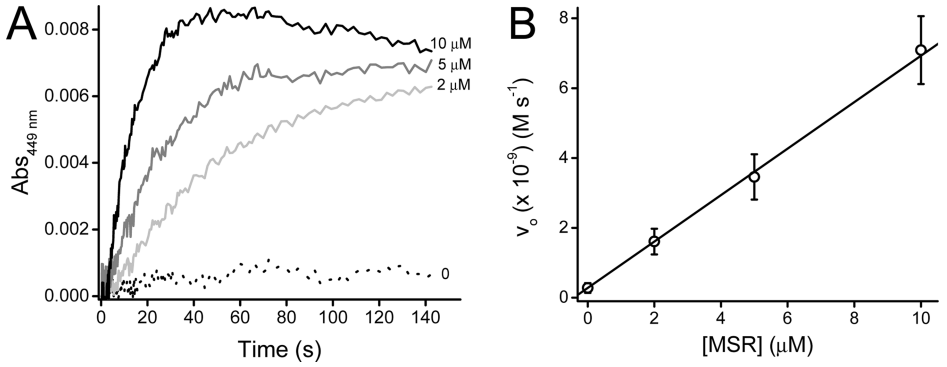

Figure 5. Stopped-flow analysis of the formation of Fe(II)-CBS in the presence of MSR and CO. (A) Kinetic traces at 449 nm were obtained from the UV−visible spectra in Figure 4, with Fe(III)-CBS (1 μM) and CO (~500 μM) in phosphate buffer (0.1 M, pH 7.4 with 0.1 mM DTPA) at 25 °C under anaerobic conditions, in the absence (···) or presence of various concentrations of NADPH-reduced MSR (as indicated). Traces shown are the means of duplicates for a representative experiment. (B) Initial rates were obtained from linear fits in the first 15 s. Each value is the mean ± standard deviation ($n \geq 5$) of data obtained on two different days.

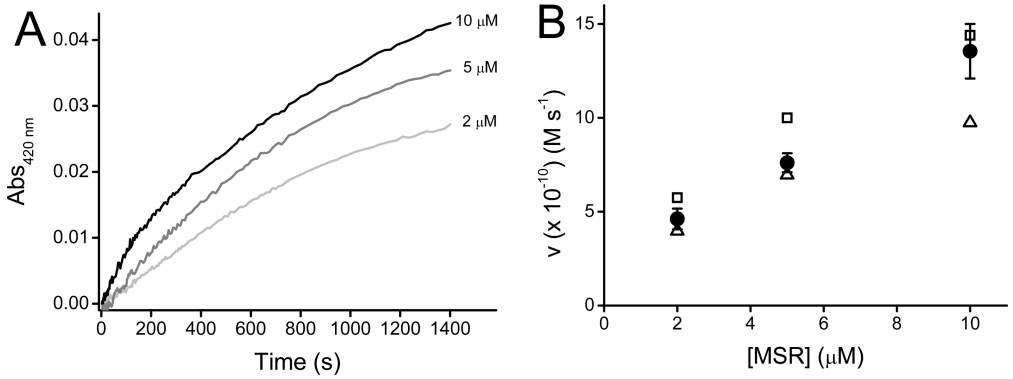

Figure 6. Stopped-flow analysis of the formation of Fe(II)CO-CBS in the presence of MSR and CO. (A) Kinetic traces at 420 nm were obtained from the UV−visible spectra in Figure 4, with Fe(III)-CBS (1 μM) and CO (~500 μM) in phosphate buffer (0.1 M, pH 7.4 with 0.1 mM DTPA) at 25 °C under anaerobic conditions, in the presence of various concentrations of NADPH-reduced MSR (as indicated). Representative traces from two independent experiments are shown. (B) Rates were obtained from linear fits of data at 40−80 or 100−250 s, depending on the trace. Each experimental value (●) is the mean ± standard deviation ($n \geq 3$) of data from two independent experiments. Empty symbols represent data from computer simulations performed for the reaction sequence shown in eqs 4 and 5 using the initial concentrations of CBS and MSR and k_4 and k_{-4} values of 9.3×10^2 and 1.2×10^5 M^{-1} s^{-1}, respectively. k_5 was assumed to be 5×10^{-3} s^{-1} (△) or 8×10^{-3} s^{-1} (□).

449 nm (Figure 5A) were analyzed using an initial rate approach, assuming that the concentration of the reagents remained constant and equaled the initial concentration. The difference absorption coefficient at 449 nm was determined to be 63900 ± 5020 M^{-1} cm^{-1} (n = 11) using dithionite as a reductant (not shown). The initial rate of Fe(II)-CBS formation, determined from the first 15 s of the kinetic traces, increased linearly with MSR concentration (Figure 5B). When the slope of the plot was divided by the concentration of CBS, the rate constant k_4 for the reduction of Fe(III)-CBS was determined to be $(9.3 \pm 2.5) \times 10^2$ M^{-1} s^{-1} (pH 7.4, 25 °C).

Although conversion of Fe(II)-CBS to Fe(II)CO-CBS (eq 5) is slow relative to the initial reduction step (eq 4), only ~15% of the initial CBS concentration formed the Fe(II)-CBS intermediate. This low yield is likely due to a relatively rapid reverse reaction, i.e., oxidation of Fe(II)-CBS to Fe(III)-CBS, consistent with the low reduction potential of CBS (−0.350 V[20]) versus that of MSR (−0.227 V[34]). On the basis of the overall $\Delta E^{\circ\prime}$ for reduction of CBS by MSR, a $\Delta G^{\circ\prime}$ value of 2.8 kcal mol^{-1} and an equilibrium constant of 7.7×10^{-3} were calculated for eq 4, representing $[Fe(II)\text{-}CBS][MSR_{ox}]/([Fe(III)\text{-}CBS][MSR_{red}])$, which correlates with the concentration quotient, $\sim 8 \times 10^{-3}$, that can be estimated from the amount of Fe(II)-CBS formed (Figure 5A). From the equilibrium constant and the forward reaction (k_4 = 9.3×10^2 M^{-1} s^{-1}), the reverse rate constant, k_{-4}, was estimated to be 1.2×10^5 M^{-1} s^{-1}.

Analysis of the formation of Fe(II)CO-CBS at 420 nm yielded an average k_{obs} value of $(9.7 \pm 3.0) \times 10^{-4}$ s^{-1} (Figure 6A). For a mechanism involving the initial formation of Fe(II)-CBS in a relatively rapid equilibrium step followed by reaction with CO (eqs 4 and 5), the net k_{obs} for Fe(II)CO-CBS formation is given by k_5 multiplied by the fraction of CBS that is in the reduced state. This fraction depends on the concentrations of reduced and oxidized MSR and CBS and on rate constants k_4 and k_{-4}, changes during the time course of the reaction, and is difficult to evaluate, because it involves second-order steps in the forward and reverse directions. In addition, regeneration of reduced MSR is likely to occur according to the relatively high rates reported for reduction of MSR by NADPH.[36,50] Assuming that the fraction of reduced CBS was ~15%, k_5 can be roughly estimated to be $\sim 6 \times 10^{-3}$ s^{-1} (~500 μM CO), in reasonable agreement with the value of 3.1×10^{-3} s^{-1} obtained for Fe(II)CO-CBS formation when dithionite was used as the reductant (Figure 2). To complement the data analysis, the rates of Fe(II)CO-CBS

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formation were measured from the slopes of the linear portions of kinetic traces at 420 nm (40–80 or 100–250 s, depending on the trace) divided by the difference extinction coefficient of 57360 M^{-1} cm^{-1} (Figure 6B). The rates increased with MSR concentration. Kinetic simulations performed with GEPASI using the initial concentrations of CBS and MSR and k_4 and k_{-4} values of 9.3×10^2 and 1.2×10^5 M^{-1} s^{-1}, respectively, showed good agreement with experimental values when k_5 was assigned a value between 5 and 8×10^{-3} s^{-1} (Figure 6B).

Kinetics of Reaction of Fe(II)CO-CBS with O_2. As observed previously, exposure of Fe(II)CO-CBS to air resulted in its rapid reoxidation to Fe(III)-CBS in a process concomitant with recovery of enzyme activity.[32] The kinetics of the O_2-dependent Fe(II)CO-CBS oxidation were characterized by stopped-flow spectrophotometry. For this, Fe(III)-CBS was reduced with dithionite in the presence of SOD[31] and saturated with CO. Formation of Fe(II)CO-CBS was confirmed spectrophotometrically (Figure S2 of the Supporting Information). As shown in Figure 7, when Fe(II)CO-CBS was mixed

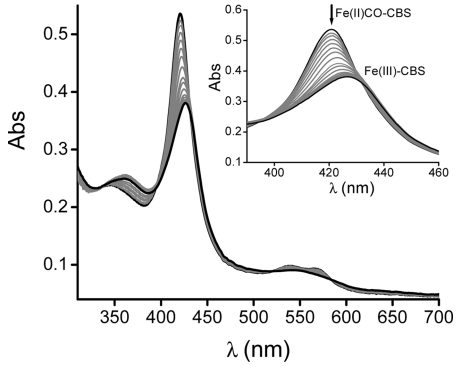

Figure 7. Oxidation of Fe(II)CO-CBS by O_2. An anaerobic solution of Fe(II)CO-CBS (5 μM) was mixed with O_2 (0.625 mM) in the presence of CO (500 μM) and SOD (0.25 μM monomer), in phosphate buffer (0.1 M, pH 7.4, 0.1 mM DTPA) at 25 °C. UV–visible absorption spectra were registered from 0.75 to 1425 s. Selected spectra (from top to bottom at 420 nm) recorded 2.25, 3.75, 5.25, 8.25, 12.75, 20.25, 30.75, 52.5, 100.5, 150, 300, 603, 801, 1017, 1209, and 1425 s after mixing are shown.

with excess O_2, it was converted to Fe(III)-CBS. To estimate the rate constants associated with the reoxidation process, both multiwavelength global fits and single-wavelength kinetic traces were analyzed, with similar results. The decrease in absorbance at 420 nm was multiphasic and was studied in different time frames (Figure 8). The first phase occurred with a k_{obs} of >0.2 s^{-1} (half-time of <3 s), and its amplitude represented <25% of the absorbance change. The second phase had a k_{obs} of $\sim 10^{-2}$ s^{-1} and an amplitude of ~50%. The third phase had a k_{obs} of $\sim 10^{-3}$ s^{-1} and an amplitude of <25%. The first and third phases did not show a clear pattern of O_2 dependence and were not studied further. The second phase was associated with the largest amplitude change, and its k_{obs} showed evidence of saturation behavior (Figure 9). From the hyperbolic fit, the maximal k_{obs} was determined to be 0.033 ± 0.013 s^{-1} and the O_2 concentration at the half-maximal k_{obs} was 0.10 ± 0.07 mM.

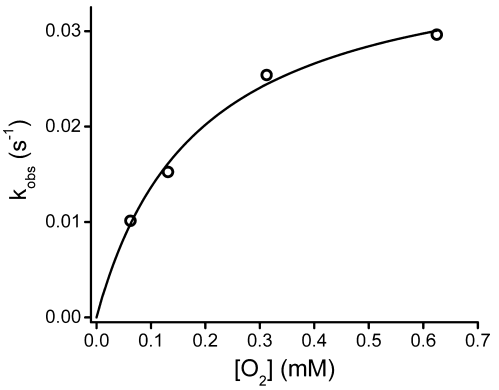

Figure 9. Dependence of k_{obs} for reaction of Fe(II)CO-CBS with O_2. Kinetic spectra were obtained as in Figure 7 at increasing O_2 concentrations (0.0625–0.625 mM). Representative k_{obs} values for the second phase of the reaction obtained from the global fit to a double-exponential function of two independent experiments recorded for 148 s are shown. The black line represents the best fit to a hyperbolic function.

Lack of Interaction of CBS with Exogenous H_2S. Because CBS is a source of H_2S in mammals,[51−53] we tested the hypothesis that its heme might have a role as a sulfide sensor. However, the presence of up to 13 mM exogenous H_2S had no effect on the enzymatic activity of Fe(III)-CBS or Fe(II)-CBS (Figure S3 of the Supporting Information). In addition, exposure to 0.5–3.2 mM H_2S had no effect on the absorption spectra of Fe(III)-CBS (Figure S4 of the Supporting Information). With Fe(II)-CBS, the presence of either oxidizing contaminants or decomposition products in the H_2S solution resulted in oxidation to Fe(III)-CBS (data not shown), but when dithionite (280 μM) was added to the H_2S stock solution (0.13 M), no changes in the absorption spectra of Fe(II)-CBS were observed in the presence of 0.1–4.1 mM H_2S

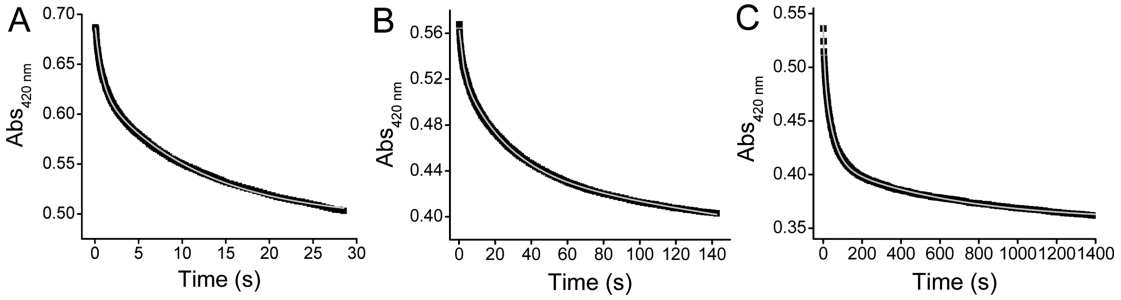

Figure 8. Kinetic traces of the reaction between Fe(II)CO-CBS and O_2. The absorbance traces at 420 nm as a function of time are shown up to (A) 28, (B) 142, and (C) 1400 s. The data were obtained from the UV–visible spectra in Figure 7. The gray lines represent the best fits to a double-exponential function (A and C) and double-exponential and straight line functions (B).

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Table 1. Reactions Involved in the Reduction and Reoxidation of the Heme in CBS

reaction	rate constant[a]	ref
$S_2O_4^{2-} \rightleftharpoons 2SO_2^{\bullet-}$	2.5 s^{-1} (f), 1.8 × 10^9 M^{-1} s^{-1} (r)[b]	45, 48
Fe(III)-CBS + $SO_2^{\bullet-}$ → Fe(II)-CBS + SO_2	(9.5 ± 0.2) × 10^6 M^{-1} s^{-1}	this study
Fe(III)-CBS + MSR_{red} ⇌ Fe(II)-CBS + MSR_{ox}	(9.3 ± 2.5) × 10^2 M^{-1} s^{-1} (f), 1.2 × 10^5 M^{-1} s^{-1} (r)[b]	this study
Fe(II)-CBS + O_2 → Fe(III)-CBS + $O_2^{\bullet-}$	(1.13 ± 0.05) × 10^5 M^{-1} s^{-1}	31
Fe(II)-CBS + CO → Fe(II)CO-CBS	(3.1 ± 0.4) × 10^{-3} s^{-1} at 1 mM CO[c]	this study
Fe(II)CO-CBS + O_2 → Fe(III)-CBS + products	0.033 ± 0.013 s^{-1} at infinite O_2[d]	this study
Fe(III)-CBS + H_2S → products	not detected	this study
Fe(II)-CBS + H_2S → products	not detected	this study

[a]Rate constants at pH 7.4 and 25 °C except for those for the homolysis of dithionite, which are reported at pH 6.5.[45,48] [b]f, forward; r, reverse. [c]Pseudo-first-order rate constant (k_{obs}) obtained at 1 mM CO (see Discussion). [d]Multiphasic process, limiting pseudo-first-order rate constant (k_{obs}) of the major phase, that has a hyperbolic dependence on oxygen concentration (see Results and Discussion).

(Figure S5 of the Supporting Information). Thus, no evidence of potential feedback regulation of CBS by H_2S was found.

DISCUSSION

In this study, we have characterized the kinetics of reductive carbonylation of the heme in CBS using a chemical (dithionite) and a biochemical (MSR and NADPH) reductant (Table 1 and Scheme 1). The kinetics of binding of CO to Fe(II)-CBS, obtained using very low dithionite concentrations and 1 mM CO at pH 7.4, were slow and monophasic, with a k_{obs} of 3.1 × 10^{-3} s^{-1}, probably representing cysteine dissociation. This result is comparable to the time scale of PLP tautomerization from the active ketoenamine to the inactive enolimine that occurs when CO binds to Fe(II)-CBS.[17] It is also consistent with the kinetics of decay of Fe(II)-CBS to Fe(II)424-CBS, where the Cys52 ligand is replaced by a neutral ligand.[54] A previous study of the kinetics of binding of CO to CBS was conducted at pH 8.6 and reported biphasic kinetics. The fast phase increased hyperbolically with CO concentration, with a limiting k_{obs} of 0.0166 s^{-1} and a half-maximal value at 0.109 mM CO, while the slower phase was independent of CO concentration with a k_{obs} of ∼1.1 × 10^{-3} s^{-1}.[55] Another more recent study[49] reported that the kinetics of CO binding are affected by pH and by the presence of Fe(II)424-CBS. The amount and purity of dithionite used are probably also critical considerations.[56]

Reductive carbonylation of CBS in the presence of MSR exhibited a dependence on ionic strength, suggesting that electrostatic contacts are involved in protein−protein interactions. The environment around the heme pocket in CBS is electropositive, while that around the FMN cofactor in MSR is negative.[31,57] A similar ionic strength maximum at 220 mM has been reported for the interaction of MSR and methionine synthase,[33] with acidic residues in MSR being critical.[36,57] The unfavorable redox potentials between MSR and the CBS heme lead to a rather small rate constant for heme reduction of CBS by MSR (9.3 × 10^2 M^{-1} s^{-1}) coupled with a large reverse rate constant (1.2 × 10^5 M^{-1} s^{-1}). An alternative reductase with a redox potential lower than that of MSR is likely to be more effective at reducing CBS. For example, the cytosolic human novel reductase 1, also in the same reductase family but of unknown biological function, has an FMN semiquinone to hydroquinone reduction potential of −0.305 V[58] and is expected to be a better reductant of the heme in CBS.

In contrast to the monophasic reaction of Fe(II)-CBS with O_2 to Fe(III)-CBS and superoxide, which shows a linear dependence on O_2 concentration (1.13 × 10^5 M^{-1} s^{-1}), consistent with an outer sphere process,[31] reoxidation of Fe(II)CO-CBS was multiphasic and involved a step with a hyperbolic dependence on O_2 concentration. This multiphasic behavior is compatible with several parallel or consecutive reactions and suggests the coexistence of several heme species. In principle, reoxidation of Fe(II)CO-CBS to Fe(III)-CBS may occur directly or may involve the intermediate formation of Fe(II)-CBS. In addition, pentacoordinate Fe(II)5c-CBS may also be involved as an intermediate during the reoxidation process and, at relatively long time frames, Fe(II)424-CBS. The kinetics of reactions of these latter two species with O_2 are not known. We hypothesize that the phase exhibiting saturation behavior reflects a step prior to reaction with O_2 being limiting, e.g., dissociation of CO (0.033 s^{-1}).

Studies with cell cultures and tissues suggest that the reductive carbonylation of CBS occurs *in vivo*, because an increase in CO concentration decreases the concentration of cystathionine, presumably because of inhibition of CBS.[59,60] The interaction of CBS with CO is proposed to be involved in the regulation of cerebral vasodilation triggered by hypoxia.[61] According to this model, a diminished O_2 availability leads to a decreased level of production of CO by heme oxygenase, which in turn prevents downregulation of CBS by CO, resulting in greater H_2S production and consequently an increased level of vasodilation. This hypothesis implies that the heme must be in the reduced ferrous state under normoxic conditions for CBS to be inhibited by CO; therefore, the relatively high rate of reoxidation by O_2 must be countered by some efficient reductive process(es).[31]

Assigning a sensor role to the heme in CBS heme depends critically on the feasibility of its reduction to the ferrous state, because the ferric form is stable and does not appear to interact with ligands that could result in the inhibition of enzymatic activity.[23,24] Indeed, as shown herein, the heme does not interact with H_2S, itself a product of the enzymatic activity, despite the fact that a cysteine thiolate serves as a heme ligand in the ferric and ferrous states. Considerations of the possible roles of the CBS heme need to take into account both reduction to the ferrous state and its subsequent reaction with CO to form the inactive Fe(II)CO-CBS species or with O_2 to form Fe(III)-CBS and $O_2^{\bullet-}$. The finding that the heme in CBS can be reversibly reduced by a biochemical system reported previously[32] and characterized in more detail in this study supports the possibility of a sensor role for this cofactor and provides a mechanism for cross talk among the CO, H_2S, and $O_2^{\bullet-}$ signaling pathways.

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■ ASSOCIATED CONTENT

Supporting Information

Figures showing the control experiments of heme reduction by MSR, UV−visible absorption spectra of Fe(III)- and Fe(II)-CBS in the presence of CO or H_2S, and enzymatic activity of CBS in the presence of H_2S. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*B.A.: Laboratorio de Enzimología, Facultad de Ciencias, Iguá 4225, 11400 Montevideo, Uruguay; phone and fax, +598-2-5250749; e-mail, beatriz.alvarez@fcien.edu.uy. R.B.: Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109-0600; phone, (734) 615-5238; e-mail, rbanerje@umich.edu.

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Notes

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■ ABBREVIATIONS

CBS, cystathionine β-synthase; Fe(III)-CBS, CBS with ferric heme; Fe(II)-CBS, CBS with ferrous heme; CO, carbon monoxide; Fe(II)CO-CBS, CBS with ferrous heme and CO as a ligand; Fe(II)424-CBS, CBS with ferrous heme and a neutral ligand replacing cysteine; Fe(II)5c-CBS, pentacoordinate ferrous CBS; PLP, pyridoxal 5′-phosphate; SOD, superoxide dismutase; DTPA, diethylenetriaminepentaacetic acid; MSR, methionine synthase reductase.

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