Low sulfide levels and a high degree of cystathionine β-synthase (CBS) activation by S-adenosylmethionine (SAM) in the long-lived naked mole-rat

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Abstract

Hydrogen sulfide (H2S) is a gaseous signalling molecule involved in many physiological and pathological processes. There is increasing evidence that H2S is implicated in aging and lifespan control in the diet-induced longevity models. However, blood sulfide concentration of naturally long-lived species is not known. Here we measured blood sulfide in the long-lived naked mole-rat and five other mammalian species considerably differing in lifespan and found a negative correlation between blood sulfide and maximum longevity residual. In addition, we show that the naked mole-rat cystathionine β-synthase (CBS), an enzyme whose activity in the liver significantly contributes to systemic sulfide levels, has lower activity in the liver and is activated to a higher degree by S-adenosylmethionine compared to other species. These results add complexity to the understanding of the role of H2S in aging and call for detailed research on naked mole-rat transsulfuration.

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1. Introduction

Hydrogen sulfide (H2S) is a gasotransmitter playing a role in many physiological and pathological processes e.g. inflammation, apoptosis, cellular energetics, vascular contractility. Known molecular mechanisms underlying H2S effects include activation of ion channels, regulation of second messengers (cAMP, cGMP, free calcium) levels, and protein sulfhydration [1]. In mammals, H2S is produced mainly by two enzymes of the evolutionarily conserved transsulfuration pathway, cystathionine β-synthase (CBS, EC 4.2.1.22) and cystathionine γ-lyase (CSE, EC 4.4.1.1), as well as 3-mercaptopyruvate sulfurtransferase (MST, EC 2.8.1.2).

CBS is a key regulatory enzyme at the intersection of the transsulfuration pathway and methionine cycle, controlling the flux of methionine into transsulfuration (Fig. 1B). In the canonical reaction CBS catalyses condensation of homocysteine and serine to form cystathionine and water. However, when cysteine is used instead of serine, cystathionine and H2S are produced. CBS is a pyridoxal 5′-phosphate and heme dependent enzyme consisting of three structural domains: (i) N-terminal heme binding domain, (ii) catalytic core, and (iii) C-terminal regulatory domain with an autoinhibitory function (Fig. 1A). Binding of a universal methyl group donor S-adenosylmethionine (SAM) to the regulatory domain activates and stabilizes the enzyme [2,3].

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Although there is increasing evidence that H$_2$S is implicated in aging and lifespan control, its exact role in these processes is still not clear. Exogenous H$_2$S increases lifespan in Caeurohabditis elegans [4]. Moreover, CBS is required for the life-prolonging effect of caloric restriction in Drosophila [5], and increased H$_2$S production in models for diet-induced longevity was observed [6]. In contrast, a decrease in CBS protein levels and activity in response to methionine and isocaloric protein restriction, respectively, was shown [2,7].

Importantly, sulfide concentration in naturally long-lived species remains unknown. A measure of longevity employed in this study is maximum longevity residual, which represents the relationship of the observed maximum lifespan of the species to its expected, body size-based lifespan calculated with the mammalian allometric equation [8]. Human and naked mole-rat belong to species with the highest maximum longevity residual. The naked mole-rat (Heterocephalus glaber) is a eusocial subterranean rodent native to East Africa. It has become the focus of increased attention as a model for aging and cancer research due to its extremely long lifespan [9]. In addition to high maximum longevity residual, it has the highest maximum residual obtained from the AnAge database (http://genomics.senescence.info/species accessed on 14.09.2015). Of note, there are differences in CBS activity in the liver significantly contributes to the circulating H$_2$S levels [11], we comparatively analyse the naked mole-rat CBS gene.

F. micklemi and F. mechowii are maintained at the animal facilities of the Department of General Zoology, University of Duisburg-Essen, Germany. They are housed as family groups in glass terraria on horticultural peat and fed ad libitum with carrots and potatoes every day, apples every second day, and grain and lettuce once a week. Room temperature and humidity is kept constant at 24 $\pm$ 1 °C and 40 $\pm$ 3%, respectively. Sampling was approved by Landesamt für Natur-, Umwelt- und Verbraucherschutz Nordrhein-Westfalen (Az. 84-02.04.2013.A164).

Guinea pigs (Cavia porcellus, Dunkin Hartley HsdDhl:DH) were purchased from Harlan Laboratories, IL, USA, and were maintained at Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany under room conditions in plastic cages with litter and hay as bedding. The range of the room temperature is 18–20 °C and of the humidity 40–50%. Fresh food is given daily and includes carrot, cucumber, salad, apples, and dry feed. Sampling was approved by the local ethics committee of the "Landesamt für Gesundheit und Soziales", Berlin, Germany (G02217/12).

Mice (Mus musculus, C57BL/6) were maintained at the Center of Sepsis Control and Care (Jena University Hospital, Jena, Germany). They were maintained under artificial day-night conditions at room temperature, and received a standard diet and water ad libitum. Animals were randomly selected for each experiment. Sampling was approved by Thüringer Landesamt für Verbraucherschutz (02-035/12).

2.2. Human samples

Blood samples were obtained from healthy volunteers of European origin after written informed consent and approval by the Jena University Ethics Committee (3624-11/12).

2.3. Quantification of sulfide in whole blood

Sulfide was measured by GC/MS after extractive alkylation using a bis-pentafluorobenzyl derivative. The method and its calibration were described in detail in [12]. 25 μl blood was used and the volume of the reaction mixture was adjusted accordingly. Species and sampling information is listed in Table 1.
no entries for *F. mechowii* and *F. micklemi* in the AnAge database. Therefore, maximum longevity residual for *F. mechowii* was calculated with the allometric equation provided by the database: 
\[ t_{\text{max}} = 4.88t_{\text{m}} + 1.35 \]. According to the species’ body weight, *F. mechowii* was 21.0 years breeding female, and the mean body weight of *F. mechowii* females is 250 g (unpublished data).

*F. micklemi* is a small bathyergid occurring in Western and Southern Zambia. The maximum lifespan of this species is not yet established because it has been assigned species status only relatively recently [13] and has been bred in laboratories only since 2008. However, *F. micklemi* is very closely related to the better studied *F. anselli* and *F. kafuensis*, with all three species belonging to the same “Fukomys micklemi” clade according to [14]. *F. micklemi* interbreeds with *F. anselli* in the lab (own unpublished data), and both species are nearly indistinguishable regarding their body measures and biology including mating and social system. We therefore used the maximum longevity residuals of *F. anselli* as the currently best available approximation for *F. micklemi*.

### 2.4. Cell culture

HCT116 cells were purchased from ECACC through Sigma (Sigma-Aldrich, St. Louis, MO, USA). Cells were grown at 37 °C in the presence of 5% CO2 in McCoy’s Medium (Gibco, Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% FBS. HEK293-EBNA cells were a kind gift from Dr. Christoph Kaether (Leibniz Institute for Age Research-Fritz Lipmann Institute, Jena, Germany). Cells were grown at 37 °C in the presence of 5% CO2 in DMEM Medium (Gibco, Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% FBS.

### 2.5. RT-PCR

RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. 900 ng total RNA was used for reverse transcription with QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). CBS and *GAPDH* were amplified with the use of primers listed in Table 2 and following PCR conditions: 95 °C 1 min, followed by 29 cycles with 95 °C 30 s, 59 °C 30 s, 72 °C 1 min, and final extension at 72 °C for 10 min. PCR products were analyzed by agarose (1% w/v) gel electrophoresis.

### 2.6. RNA-seq

For library preparation 1 μg of total RNA was introduced into Illumina’s (Illumina, San Diego, CA, USA) TrueSeq RNA sample prep kit v2 following the manufacturer’s instruction. Quality checking and quantification of the library was done using an Agilent Bioanalyzer 2100 in combination with an Agilent DNA 7400 kit (Agilent Technologies, Inc., CA, USA). The library was sequenced on a HiSeq2500 in high-output, 50 bp single-read mode. SBS sequencing chemistry v3 was used (Illumina, San Diego, CA, USA). Read information were extracted in FastQ format using bcl2fastq v1.8.4 (supported by Illumina). The sequencing approach resulted in 57,815,446 single-end reads. The reads were mapped to the human genome (hg19) taken the RefSeq [15] annotation (release 64) into account using tophat v1.4.1 [16]. The mapping result was introduced into htseq-count [17] using the annotation as mentioned above to count reads per gene.

### 2.7. Test for positive selection

Orthologous genes were determined by best-bidirectional-blast-hits. Per species (*Mus musculus*, *Rattus norvegicus*, *Mesocricetus auratus*, *Cricetus griseus*, *Nannospalax galili*, *Cavia porcellus*, *F. mechowii*, *F. micklemi*, *Canis lupus*, *Pan troglodytes*, *Homo sapiens*, *Oryctolagus cuniculus*) that splice variant was chosen that showed highest similarity to naked mole-rat transcript (XM_004885703). Codon alignments were conducted using prank [18]. The alignments were filtered by gblocks [19]. Next, PAML’s [20] branch-site test of positive selection was applied using naked mole-rat as foreground branch.

### 2.8. Plasmids and site-directed mutagenesis

The CBS CDS of naked mole-rat (XM_004885703), *F. mechowii* (KR028540), human (NM_000071), C431L and C431S variants, and human core with naked mole-rat regulatory domain were synthesized and cloned into pCMV6-AC plasmid by Blue Heron.

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### Table 1

**Species and sampling information for sulfide measurement in blood.**

| Species | Mean age ± SD | Number of females | Number of males | Source of blood | Anesthesia |
|---------|---------------|-------------------|-----------------|----------------|------------|
| Hgl     | 44 months ± 5 | 2 Breeder 3 Non-breeder | 3 Breeder 2 Non-breeder | Heart puncture/vein | Isoflurane |
| Fme     | 56 months ± 15 | 9 Breeder 2 Non-breeder | 7 Breeder 9 Non-breeder | Vein | Retamine and xylazine |
| Fmi     | 31 months ± 15 | 2 Breeder 6 Non-breeder | 2 Breeder 9 Non-breeder | Vein | Retamine and xylazine |
| Mmu     | 5 years ± 5 | 4 Breeder 6 Non-breeder | 17 Breeder 9 Non-breeder | Retro-orbital puncture | Isoflurane |
| Cpo     | 12 months | 1 Breeder 3 Non-breeder | Heart puncture | Medetomidine, midazolam, and fentanyl |
| Hsa     | 45 years ± 11 | 5 Breeder 9 Non-breeder | Vein |

Hgl – *H. glaber*, Fme – *F. mechowii*, Fmi – *F. micklemi*, Cpo – *C. porcellus*, Mmu – *M. musculus*, Hsa – *H. sapiens*.

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### Table 2

**Primer sequences used for RT-PCR analysis, site-directed mutagenesis, and cloning.**

| Gene | Primer names and sequences | CBS qPCRhuman_1F | CBS qPCRhuman_1R | TGGCTCAAGGACTTTGTTT |
|------|---------------------------|------------------|------------------|----------------------|
| CBS  | cbs_qPCRhuman_1F: ATGCTGATCGCGCAAGAG; cbs_qPCRhuman_1R: TGGCTCAAGGACTTTGTTT |
| GAPDH| hs_GAPDH_1F: ACGCGCACTCATCTCCCATATCTGGGAA; hs_GAPDH_1R: GTATCGACAGGGGCGGAGAG |
| Mmu  | mcbscDNA_EcoRI_1F: TCTAGGAATCTCGACCATCTCATCTCTCGGTTT; mcbscDNA_EcoRI_1R: CTAGTTTAAATCTCGATGGGTAGAAGCTGGTAG |
| Hgl  | CBS CT234TC | nmrCBSMat_1F: CCCACTCGCACCTCCAAGCAACCAT; nmrCBSMat_1R: GAGGCTGCTGCTCAGGTAGGAGGGG |
| Fme  | CBS CT234TC | nmrCBS1412S_1F: TTGCGGCCACGGGAGGCAGCAGCCACTAC; nmrCBS1412S_1R: ATGCCGGAGGCTGCTGAGCAGCAGCCACTAC |
| Fme  | CBS T355C T358G | CBSmechToAnsel_2F: GAGACAGAGACCCCGGGGACCTCT; CBSmechToAnsel_2R: AGATCGAGGCAGGCGGTGCTTCTT |

Hgl – *H. glaber*, Fme – *F. mechowii*, Fmi – *F. micklemi*, Mmu – *M. musculus*. 

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| Fmi     | 31 months ± 15 | 2 Breeder 6 Non-breeder | 2 Breeder 9 Non-breeder | Vein | Retamine and xylazine |
| Mmu     | 5 years ± 5 | 4 Breeder 6 Non-breeder | 17 Breeder 9 Non-breeder | Retro-orbital puncture | Isoflurane |
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Hgl – *H. glaber*, Fme – *F. mechowii*, Fmi – *F. micklemi*, Cpo – *C. porcellus*, Mmu – *M. musculus*, Hsa – *H. sapiens*.
Biotechnology, Inc. (Bothell, WA, USA). The naked mole-rat L412C and L412S variants, and the *F. mickelmi* CDS (KR028541) were generated by site-directed mutagenesis using the naked mole-rat or *F. mechowii* pCMV6-AC constructs as a template. Primers containing desired mutations were designed with the primer-design program PrimerX (http://www.bioinformatics.org/primerx). Accessed 3 September 2014). The template replicated with Pfu/Psp DNA polymerase (GeneON, Ludwigshafen am Rhein, Germany) was digested with DpnI (NEB, Ipswich, MA, USA).

CDS with naked mole-rat core and human regulatory domain was created by exchanging regulatory domains between human and naked mole-rat pCMV6-AC constructs. Eco47III (Thermo Scientific, Waltham, MA, USA) (restriction site spanning nucleotides 1103 to 1108 of human CBS coding sequence) and Pmel (Thermo Scientific, Waltham, MA, USA) (restriction site in the multiple cloning site) were used to cut plasmids. The fragments were separated on a 1% agarose gel, purified with GeneJET Gel Extraction Kit (Thermo Scientific, Waltham, MA, USA) and digested with DpnI (NEB, Ipswich, MA, USA).

Mouse CDS (NM_178224) was amplified using mouse liver cDNA from MTC Multiple Tissue cDNA Panels (Clontech, Mountain View, CA, USA) as a template. Primers contained EcoRI and Pmel restriction sites. PCR product and pCMV6-AC plasmid were digested with EcoRI (NEB, Ipswich, MA, USA) and Pmel (Thermo Scientific Waltham, MA, USA) and purified from 1% agarose gel with GeneJET Gel Extraction Kit (Thermo Scientific, Waltham, MA, USA). PCR product was ligated into the plasmid with the Quick Ligation Kit (NEB, Ipswich, MA, USA).

All plasmids were amplified in *E.coli* TOP10 cells (Life technologies, Carlsbad, CA, USA) and purified with a Plasmid Maxi Prep Kit (Qiagen, Valencia, CA, USA). Plasmid sequences were confirmed by Sanger sequencing using BigDye Terminator v3.1 Sequencing Standard Kit (Applied Biosystems, Foster City, USA) and ABI3730xl DNA Analyzer. All primer sequences are listed in Table 2.

2.9. CBS activity assay

HCT116 cells were transiently transfected with FugeneHD (Promega, Madison, WI, USA) according to manufacturer’s protocol using 4:1 reagent:DNA ratio. Cells were harvested 24 or 48 h after transfection and cell pellets were lysed in STEN buffer (50 mM Tris HCl, 150 mM NaCl, 2 mM EDTA, 0.2% NP40, pH 8) supplemented with Halt Protease Inhibitor Cocktail (Thermo Scientific, Waltham, MA, USA). Protein concentration was measured with Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA).

CBS activity was assayed by measurement of *H*₂*S* production with an *H*₂*S* specific probe 7-azido-4-methylcoumarin (AzMC) (Sigma-Aldrich, St. Louis, MO, USA). The assay was designed after [21]. The reaction mixture contained: 200 mM Tris HCl pH 8.0, 5 μM pyridoxal 5′-phosphate, 10 mM glutathione, 0.5 mg/mL BSA, 50 μM AzMC and cell lysate. Always the same amount of protein was used within one assay. The reaction mixture was incubated with or without SAM (0.5 mM) for 60 min at 37 °C. Always cell lysate from one transfection was divided and used for the measurement in the absence and the presence of SAM. The fluorescence at 450 nm (exc. 365 nm) was read with Infinite M1000 microplate reader (Tecan, Männedorf, Switzerland). Data were normalized to mock transfected cells. Fold activation was calculated by dividing CBS activity with SAM by CBS activity without SAM.

2.10. *H*₂*S* producing CBS activity in the liver

Liver tissue from 8 non-breeding naked mole-rats and 8 mice (equal number of both sexes, mean age was 40 months ± 6 and 8 months ± 0.5, respectively) was cut into pieces and frozen immediately after collection and kept at −20 °C until usage. Liver pieces were disrupted in Tissue Lyser LT (Qiagen, Valencia, CA, USA) using stainless steel beads in 1 ml STEN buffer supplemented with proteinase inhibitor mix. Tissue lysate was incubated 30 min on ice and centrifuged at 13,000 rpm for 8 min. The supernatant was dialysed in Slide-A-Lyzer Dialysis Cassettes, 7 K MWCO (Thermo Scientific, Waltham, MA, USA) overnight at 4 °C in STEN buffer with one buffer change after 2 hours. Protein concentration measurement and CBS activity assay were performed as in CBS activation assay in cell lysates, except for the addition of 2.5 mM DL-propargylglycine (Sigma-Aldrich, St. Louis, MO, USA), an irreversible inhibitor of cystathionine gamma-lyase (CSE). 260 μg protein was used in the assay and the incubation time was 90 min. Data were normalized to the reaction mixture containing water instead of liver lysate.

2.11. Statistical analysis

Unpaired Student’s *t*-test, one-way ANOVA followed by Tukey test, and Kruskal Wallis test followed by Nemenyi test were used to analyse differences in CBS activity in the liver, CBS activation, and sulfide levels in blood, respectively. A *p*-value of < 0.05 was considered to indicate statistical significance.

3. Results

3.1. Low blood sulfide levels in long-lived species

Using extractive alkylation with a bis-pentafluorobenzyl derivative we quantified sulfide concentration in whole blood of naked mole-rat, two other mole-rat species (*F. mechowii*, *F. micklemi*), mouse, guinea pig, and human. Sulfide levels differ significantly between species with human and naked mole-rat exhibiting the lowest values (Fig. 2A). Surprisingly, we found a negative correlation between maximum longevity residual and mean sulfide concentration in blood (Fig. 2B).

3.2. Low endogenous CBS activity in the naked mole-rat liver

Next, we compared endogenous CBS activity in the liver of naked mole-rat and mouse using *H*₂*S*-specific probe 7-azido-4-methylcoumarin (AzMC). We observed that *H*₂*S* producing activity of CBS is lower in naked mole-rat liver as compared to mouse (Fig. 3).

3.3. Substitution of a conserved cysteine to leucine in naked mole-rat CBS does not affect activation

As CBS is a significant contributor to endogenous *H*₂*S* production in mice [11] we screened the naked mole-rat CBS gene for signs of positive selection but failed (File S1). We noticed, however, in the regulatory domain at amino acid position 412 a cysteine > leucine substitution (C412L) present also in CBS from *F. mechowii* and *F. micklemi*. Cysteine at this position is conserved among all other analyzed vertebrates (Fig. 4). Notably in human CBS, the conversion of the corresponding cysteine to serine (*C431S*) creates a constitutively active form of the enzyme [22]. We, therefore, tested whether substitution of the conserved cysteine to leucine in the regulatory domain of naked mole-rat CBS affects the degree of activation of the enzyme in the presence of SAM. To this end, plasmids encoding either canonical or *in vitro* mutated human or naked mole-rat CBS were transfected into HCT116 human colorectal carcinoma cell line. One of the main criteria for the cell line to be used in this study was its lack of endogenous CBS expression, which – if present-would create a
background signal in the assay. Zhang et al. described HCT116 cells as being CBS mRNA free [23]. In contrast, Yamamoto et al. [24] and Szabo et al. [25] reported low and high CBS expression, respectively. In light of this contradiction, we performed RT-PCR and RNA-seq which confirmed that the transsulfuration pathway in HCT116 cells is suppressed (no CBS and negligible CTH transcripts detectable) (File S2). H2S-producing CBS activity was tested in cell lysates with the use of AzMC in the presence and in the absence of SAM. Conversion of leucine at position 412 to cysteine in naked mole-rat CBS did not affect enzyme activation (Fig. 5A). Similarly, mutation of the corresponding cysteine to leucine in human CBS did not show any effect. Mutation to serine resulted in a constitutively active enzyme in both species, which is in agreement with published data [22].

3.4. Strong activation of naked mole-rat CBS by SAM

The described experiment, however, revealed that the SAM activation level of naked mole-rat CBS (4-fold) is nearly doubled compared to human CBS (2.3-fold). CBS from F. meehowii, F. micklemi, Fme – F. meehowii, Cpo – C. porcellus, Mmu – M. musculus, Hsa – H. sapiens.

4. Discussion

The data presented in this study reveal an intriguing negative correlation between blood sulfide levels and maximum longevity residual. In the light of reported increase of transsulfuration activity and high H2S levels in diet-induced longevity models [5,6],
this observation is unexpected and suggests that the role of H2S in natural and diet-induced longevity is different.

Given the plethora of reported H2S effects, this finding is difficult to interpret. H2S is elevated in cardiovascular [26] and rheumatic disease [27], which suggests that the low sulfide levels may be beneficial. In addition, low CBS activity and low sulfide levels in naked mole-rat may contribute to cancer resistance in this species, as it has been shown that H2S is promoting both tumor growth and vascularisation [28]. However, the vast majority of studies report beneficial effects of increased H2S levels and most translational approaches aim to develop H2S-delivering therapeutics [1].

The low sulfide concentration in naked mole-rat blood is consistent with our findings that H2S producing activity of CBS is lower in naked mole-rat as compared to mouse. Naked mole-rats feed on the underground parts of plants. Roots and tubers show lower in naked mole-rat as compared to mouse. Naked mole-rats already elucidated [34], we experimentally studied the functional consequences of the observed substitution and found that it has no effect on the CBS activation.

However, this experiment revealed a strong activation of naked mole-rat CBS in response to SAM. While in our experiments human CBS show an activation of 2.3 fold, in the literature values ranging from 2 to 5-fold can be found [2,35]. This discrepancy can be explained by the fact that SAM binding to CBS is considerably affected by surface electrostatics [35]. Nevertheless, under normalized conditions used in this study (the same cell line transfected with CBS from different species under the control of identical promoters) naked mole-rat CBS shows consistently a higher degree of activation by SAM than human, mouse, and F. mechowii CBS.

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Fig. 5. Activation of CBS by SAM. (A) Fold activation after HCT116 transfection with expression vectors containing either the naked mole-rat CDS with leucine, cysteine and serine at position 412 (native L412, L412C and L412S, respectively) or the human CDS with cysteine, leucine and serine at position 431 (native C431, C431L and C431S, respectively). (B) Fold activation after HCT116 transfection with expression vectors coding for chimeric CBS: naked mole-rat N-terminal heme binding and catalytic domain, and human regulatory domain (Hgl core and Hsa reg) and vice versa (Hsa core and Hgl reg). (C) Fold activation of CBS from different species. Hgl – H. glaber, Fmi – F. micklemi, Fme – F. mechowii, Mmu – M. musculus, Hsa – H. sapiens. In (A) (B) (C), data represent mean ± SD of n biological replicates, p-value: * < 0.05, ** < 0.01, *** < 0.001 (one-way ANOVA followed by Tukey test). Data were normalized to the lysate from mock transfected cells.
CB activity substantially affects plasma homocysteine levels [36,37]. Since hyperhomocysteinemia is observed in many age-related diseases [38] and is a strong predictor of mortality among individuals with coronary artery disease [39], further characterization and kinetic studies of naked mole-rat CBS may shed new light on the mechanisms underlying the extremely long lifespan of this species. In summary, we found a negative correlation between blood sulfide concentration and maximum longevity residual and provide the first insights into naked mole-rat transsulfuration pathway. We report low H2S producing CBS activity in the naked mole-rat liver and a high activation of naked mole-rat CBS by SAM. In addition, we determined that the substitution of the conserved cysteine (C412L) in the regulatory domain of naked mole-rat CBS does not affect the degree of activation of the enzyme. The described features of naked mole-rat CBS call for detailed research on naked mole-rat transsulfuration pathway.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2016.01.008.

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