Biochimica et Biophysica Acta 1860 (2016) 20–27

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

The mitochondrial carnitine/acylcarnitine carrier is regulated by hydrogen sulfide via interaction with C136 and C155

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ARTICLE INFO

Article history:
Received 23 July 2015
Received in revised form 24 September 2015
Accepted 8 October 2015
Available online 14 October 2015

Keywords:
Redox biology
Carnitine
Cys modification
Membrane transport
Mutagenesis
Beta oxidation

ABSTRACT

Background: The carnitine/acylcarnitine carrier (CAC or CACT) mediates transport of acylcarnitines into mitochondria for the β-oxidation. CAC possesses Cys residues which respond to redox changes undergoing to SH/disulfide interconversion.
Methods: The effect of H_2S has been investigated on the [^{3}H]carnitine/carnitine antiport catalyzed by recombinant or native CAC reconstituted in proteoliposomes. Site-directed mutagenesis was employed for identifying Cys reacting with H_2S.
Results: H_2S led to transport inhibition, which was dependent on concentration, pH and time of incubation. Best inhibition with IC_{50} of 0.70 μM was observed at physiological pH after 30–60 min incubation. At longer times of incubation, inhibition was reversed. After oxidation of the carrier by O_2, transport activity was rescued by H_2S indicating that the inhibition/activation depends on the initial redox state of the protein. The observed effects were more efficient on the native rat liver transporter than on the recombinant protein. Only the protein containing both C136 and C155 responded to the reagent as the WT. While reduced responses were observed in the mutants containing C136 or C155. Multi-alignment of known mitochondrial carriers, highlighted that only the CAC possesses both Cys residues. This correlates well with the absence of effects of H_2S on carriers which does not contain the Cys couple.
Conclusions: Altogether, these data demonstrate that H_2S regulates the CAC by inhibiting or activating transport on the basis of the redox state of the protein.
General significance: CAC represents a specific target of H_2S among mitochondrial carriers in agreement with the presence of a reactive Cys couple.

1. Introduction

Besides the known lethal effects of hydrogen sulfide at relatively high concentrations, it is now well assessed that this gas also plays important roles in human physiology [1–5]. The range of concentrations at which physiological effects are generated is still under debate. In general, it is believed that the cellular concentration of H_2S ranges between nanomolar and three hundred micromolars depending on the measurement method and the tissues in which it is relieved [6–9]. The main known actions of this gas consist of relaxation of the cardiovascular systems (artery and veins), control of inflammation and neuroprotection, even though other roles have been postulated in liver, lung and other tissues, which however still need to be better defined [6,10]. Among other effects, H_2S has a documented effect in ameliorating reperfusion injury after ischemia [6,11,12]. The mechanism of action is, at least in some cases, mediated by interaction with sensitive protein residues such as Cys, which are reactive towards the gas. The molecular mechanisms of cellular action are poorly understood. Some proteins have already been described to interact with H_2S with important consequences on modulation of physiological functions. It is demonstrated that H_2S covalently modifies the ATP-sensitive K^+ channels causing membrane hyperpolarization and vasodilatation [13]. Cytochrome c oxidase is inhibited by H_2S [9,14]. This probably reflects the observed inhibition of mitochondrial respiration. However, other mitochondrial targets of H_2S may exist which could better explain action of this gas on mitochondrial energy metabolism. Based on present knowledge, it is expected that the best targets of H_2S would be those proteins

Abbreviations: DTE, dithioerythritol; NEM, N-ethylmaleimide; PLP, pyridoxal 5-phosphate; Pipes, 1,4-piperazinediethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CAC, mitochondrial carnitine/acylcarnitine carrier; synonyms: CACT, solute carrier family 25 member 20, symbol: SLC25A20; ORC, ornithine carrier; ANC, adenine nucleotide carrier; WT, Wild-type; NAC, N-acetylcysteine.

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http://dx.doi.org/10.1016/j.bbagen.2015.10.005
0304-4165/

whose Cys residues, on the one hand exhibit high affinity towards H_2S, on the other hand modulate the protein function upon modification.

A protein with these features might be the mitochondrial carnitine/acylcarnitine carrier. This transporter is essential for completion of the β-oxidation pathway as demonstrated by several studies performed with intact mitochondria and with native or recombinant purified proteins [15] and confirmed by the lethality of human pathology caused by defects of this protein [15,16]. CAC contains six Cys residues whose structure/function relationships have been well defined by site-directed mutagenesis, homology modeling and chemical modification. It has been demonstrated that two of these residues, C136 and C155, play a regulatory role on the protein function and, hence, to the fatty acid oxidation pathway. In particular C136 exhibits a high reactivity which is evident in terms of very low IC_{50} towards a series of chemical reagents, such as mercurial compounds [17] but also towards physiological compounds involved in the cell redox homeostasis such as glutathione. C155 is the counterpart for disulfide formation. The 2SH/S-S conversion acts as an on–off switch of the transporter, which is active only in the reduced (thiol) form [18–20]. In this work, the high affinity interaction of the CAC with H_2S has been demonstrated to occur via the couple C136–C155 suggesting a gas sensing role for these residues. The expected consequences in the regulation of energy metabolism are discussed.

2. Materials and methods

2.1. Materials

Sephadex G-75 was purchased from Pharmacia, L-[methyl-^{3}H]carnitine and L-[2,3-^{3}H]ornithine from Scopus Research BV Costerweg, [2,5′,8-^{3}H]ATP from PerkinElmer, egg-yolk phospholipids (L-α-phosphatidylcholine from fresh Turkey egg yolk), PIPES, Triton X-100, cardiolipin, L-carnitine, L-ornithine, ATP, N-dodecanoylsarcosine (sarkosyl), sodium hydrosulfide (NaHS), sodium sulfide (Na_2S), GSH, N-acetyl cysteine (NAC) and Cys from Sigma-Aldrich. All other reagents were of analytical grade.

2.2. Site-directed mutagenesis, overexpression and isolation of the CAC proteins

The previously constructed pMW7-WTratCAC recombinant plasmid was used as a template to introduce mutations in the CAC protein using complementary mutagenic primers and the High Fidelity PCR System (Roche) [21]. The PCR products were purified by the QIAEX II Gel Extraction Kit (QIAGEN), digested with NdeI and HindIII and ligated into the pMW7 expression vector. All resulting pMW7-ratCAC constructs were verified by DNA sequencing. Except for the desired base changes, all the sequences corresponded to the CAC coding sequence. The human [22] and the *Aspergillus nidulans* (fungus) CAC, amplified from an *A. nidulans* cDNA library (kindly provided by Dr. JR De Lucas) were sub-cloned into pMW 7 between NdeI and BamHI restriction sites. Bacterial over-expression was obtained using *Escherichia coli* CO214. Wild-type and mutants rat, human and *A. nidulans* CAC inclusion body fractions were isolated from *E. coli*, solubilized and purified as previously described [23].

2.3. Reconstitution of CAC, ANC and ORC in liposomes

Mitochondria has been isolated using a standard protocol that consists of: cell destruction by mechanical stress, centrifugation at low speed to remove debris and large cellular organelles, centrifugation at higher speed to isolate crude mitochondria [24].Mitochondrial rat liver extract (0.3 mg proteins in 3% Triton X-100) containing native CAC or human, rat and *A. nidulans* CAC recombinant proteins (about 0.6 μg protein) were reconstituted into liposomes by removing the detergent from the mixed micelles through an hydrophobic ion-exchange column containing the resin Amberlite XAD-4, as described previously [25]. The reconstitution mixture of mitochondrial or recombinant CAC proteins was composed of protein, 1% Triton X-100, 10 mg of egg yolk phospholipids in the form of sonicated liposomes, 10 mM Pipes at pH 7.2, 15 mM carnitine, in a final volume of 680 μL. This mixture was passed 15 times, at room temperature, through the same Amberlite column (Pasteur pipette filled with 0.5 g resin) pre-equilibrated with a buffer containing 10 mM Pipes pH 7.2 and 15 mM carnitine. In the case of ANC and ORC, the reconstitution procedure of rat liver mitochondrial extract was the same as described previously [25,26].

2.4. Transport measurements

The external substrate was removed by passing proteoliposomes through a Sephadex G-75 column (0.7 cm diameter; 15 cm height). The turbid eluate (500 μL) from the Sephadex column was collected and used for transport measurement by the inhibitor-stop method [27]. For uptake measurements, transport at 25 °C was started by adding 0.1 mM [^{3}H]carnitine or [^{3}H]ornithine or [^{3}H]ATP to proteoliposomes and stopped by the addition of 0.1 mM NEM in the case of CAC or 20 mM PLP in the case of ORC or 5 μM carboxyatractyloside in the case of ANC as previously described [28,29]. In controls, the inhibitor was added together with the labeled substrate at time zero. The experimental values were corrected by subtracting control values. Finally, the external substrate was removed by chromatography on Sephadex G-75 columns (0.7 cm diameter; 10 cm height) and intraliposomal radioactivity was measured. The measurement of CAC activity in isolated mitochondria has been performed as previously described [30].

2.5. Other methods

Amount of reconstituted protein was estimated from Coomassie blue stained SDS-PAGE gels by using the Chemidoc imaging system equipped with Quantity One software (Bio-Rad) as previously described [31].

The homology model of the human CAC was constructed using the crystallographic structure of the bovine ANC as template [32] and the computer application Swiss PDB Viewer as previously described for the CAC [33].

NaHS or Na_2S solutions were prepared as described in Ref. [34]. Measurements of sulfide content in solutions were performed using the DTNB titration method. The sulfide released by NaHS (0.90 ± 0.14 mol/mol NaHS) was very similar to that released by Na_2S (1.1 ± 0.25 mol/mol) [33]. Each experiment dealing with effects of

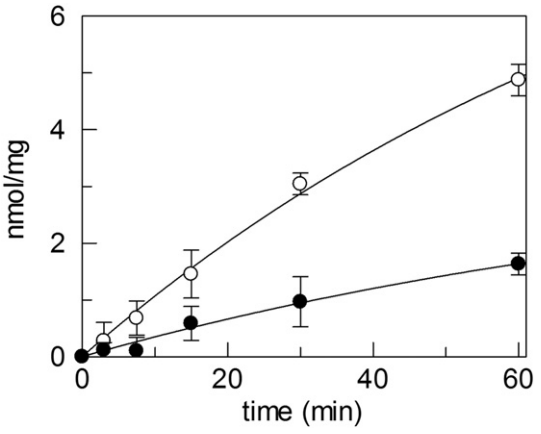

Fig. 1. Effect of H_2S on carnitine antiport activity in proteoliposomes. Transport activity of CAC was started adding 0.1 mM [^{3}H]carnitine to proteoliposomes containing 15 mM carnitine and terminated at the indicated times as described in "Materials and methods". The uptake was measured in the presence of 15 μM NaHS (●) or buffer (○). The values were the means ± SD from three experiments.