Chemico-Biological Interactions 203 (2013) 423–429

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

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

Inhibition of mitochondrial carnitine/acylcarnitine transporter by H_2O_2: Molecular mechanism and possible implication in pathophysiology

Annamaria Tonazzi [a,b,1], Lara Console [b,c,1], Cesare Indiveri [a,c,*]

[a] CNR Institute of Biomembranes and Bioenergetics, via Amendola 165/A, 70126 Bari, Italy
[b] Department of Bioscience, Biotechnology and Biopharmaceutics, University of Bari, Italy
[c] Department of BEST (Biologia, Ecologia, Scienze della Terra) Unit of Biochemistry and Molecular Biotechnology, University of Calabria, 87036 Arcavacata di Rende, Italy

ARTICLE INFO

Article history:
Received 2 December 2012
Received in revised form 14 January 2013
Accepted 31 January 2013
Available online 10 February 2013

Keywords:
ROS
Liposomes
β-oxidation
Carnitine
Mitochondria

ABSTRACT

H_2O_2 inhibits the [^{3}H]carnitine/carnitine antiport catalysed by the mitochondrial carnitine/acylcarnitine transporter reconstituted in proteoliposomes. The inhibition was reversed by dithioerythritol, N-acetyl-cysteine and L-cysteine. Inhibition time-dependence revealed a faster and a slower reaction stages with orders of reaction of 1.0 and 1.9, respectively. Inhibition was tested on mutants in which one or more of the six Cys residues had been substituted with Ser or with Val. The four replacement mutant C23S/C58S/C89S/C283S containing C136 and C155 was inhibited as the wild-type. Mutants C23V/C58V/C155V/C89S/C283S and C23V/C58V/C136V/C89S/C283S containing only C136 or C155, respectively, were inhibited at a much lower extent respect to the wild-type, while the mutant C136S/C155S in which the two Cys were substituted and the C-less protein were virtually insensitive to inhibition. DTE reversed the inhibition of the H_2O_2 sensitive proteins except that in the case of the mutants containing only C136 or C155 after long time of incubation with H_2O_2. The IC_{50} values obtained by dose–response curves of H_2O_2 inhibition were 0.17 mM for the wild-type, 0.39 mM for the four replacement mutant containing C136 and C155, 2.23 or 1.8 mM in the five replacement mutants containing the single C136 or C155, respectively. Carnitine and acetylcarnitine protected the protein from the inhibition by H_2O_2. Inhibition kinetics showed a competitive behaviour of H_2O_2 respect to carnitine. All the data concur to demonstrate that H_2O_2 interacts with C136 and C155 and completely inactivates the transporter by inducing the formation of a disulphide.

1. Introduction

H_2O_2 has several effects on proteins and, at lower concentrations, also signalling roles [1–3]. It is well established that H_2O_2, as well as other reactive oxygen species, are by products of aerobic respiration in mitochondria [1], thus it will firstly interact with proteins located in mitochondrial matrix or in the membranes and facing towards the matrix or the intermembrane space [2]. Since H_2O_2 reacts with thiol groups [3–6] an important feature of proteins, that make them sensitive towards H_2O_2, is the presence of exposed thiol residues of Cys. Thus the mitochondrial carnitine/acylcarnitine transporter (CACT), which possesses six Cys residues, represents a potential target of H_2O_2. The main role of CACT consists in catalysing the transport of acyl groups from cytosol to the mitochondrial matrix as acyl-carnitines, to allow β-oxidation [7,8]. This transport system functions according to an antiport mechanism, i.e., it translocates fatty acyl units, as acylcarnitines, into the matrix in exchange with free carnitine towards the cytosol [9]. The transport function has been well characterised in the proteoliposome experimental model, consisting of phospholipids vesicles in which the transport protein is inserted (reconstituted), mimicking the physiological environment. This model gives the important advantage to study the function of a single protein without interferences from other transporters or enzymes. Indeed proteoliposomes were reconstituted with the CACT purified from the native source of rat liver mitochondria and, later on, with the recombinant rat and human proteins obtained from over-expression in *Escherichia coli* [10,11]. Many functional and

Abbreviations: DTE, dithioerythritol; NEM, N-ethylmaleimide; Pipes, 1,4-piperazinediethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CACT, carnitine/acylcarnitine translocase; WT, wild-type; ANC, adenine nucleotide carrier; NAC, N-acetyl-L-cysteine; MTS, methanethiosulfonate; Cys, L-cysteine.

* Corresponding author. Address: Department of BEST (Biologia, Ecologia, Scienze della Terra) Unit of Biochemistry and Molecular Biotechnology, University of Calabria, via Bucci 4c, 87036 Arcavacata di Rende, Italy. Tel.: +39 0984492939; fax: +39 0984492911.
E-mail addresses: a.tonazzi@ibbe.cnr.it (A. Tonazzi), indiveri@unical.it (C. Indiveri).
[1] These authors contributed equally to this work.

0009-2797/$ - see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.cbi.2013.01.006

structural properties of the CACT have been defined. The structure of the CACT has all the typical properties of the mitochondrial carrier family, such as three repeated segments of about one hundred amino acids, each containing the signature motif PX[DE]XX[RK] [12]. The CACT proteins constitute a sub family of members expressed in various unicellular or multicellular organisms [11]. Using homology modelling and the ANC structure as template [13] the CACT structural model has been built showing that the 6 transmembrane α-helices surround a cone shaped central cavity facing the cytosol [14]. The structural model has been then validated by experiments in proteoliposomes. The site of binding of free carnitine and acylcarnitines has been identified and the molecular mechanism of translocation clarified to some extent [15–17]. The six Cys residues of CACT have been well characterised in terms of structure/function relationships by site-directed mutagenesis and chemical targeting [14,18,19]. Apparently, these residues do not play important roles in the translocation process. However, some of the six Cys residues interfere with the substrate translocation thus being potentially implied in the regulation of the transport process. Some of these residues are exposed towards the external environment and can be targeted by specific SH reagents, such as NEM or MTS. This reaction leads to transport inhibition caused by occupancy of the substrate binding site. Moreover two residues, C136 and C155 can undergo after treatment with oxidising reagents, to the formation of a disulphide which leads to complete inactivation of the protein [18]. However, so far, the study of the role of the Cys residues was limited to chemical compounds which are not involved in cell metabolism under physiological or pathological conditions. Being the exposed Cys residues possible targets of H_2O_2, the effect of this compound on the CACT has been studied and characterised in reconstituted proteoliposomes. The results highlight a potential implication of the interaction of H_2O_2 with the CACT in pathophysiology.

2. Materials and methods

2.1. Materials

L-[Methyl-^{3}H]carnitine 99% pure, 85 Ci/mmol, from Scopus Research BV, Wageningen The Netherlands, Sephadex G-75, egg-yolk phospholipids (L-α-phosphatidylcholine from fresh turkey egg yolk), Pipes, Hepes, Triton X-100, cardiolipin, L-carnitine, H_2O_2, Catalase from Sigma–Aldrich s.r.l., Milano Italy. All other reagents were of analytical grade.

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

The previously constructed pMW7-WTratCACT recombinant plasmid was used to introduce the mutations in the CACT protein as previously described [19]. The amino acid replacements were performed with complementary mutagenic primers using the overlap extension method [20] and the High Fidelity PCR System (Roche). The PCR products were purified by the QIAEX II Gel Extraction Kit (QIAGEN, La Jolla), digested with NdeI and HindIII (restriction sites added at the 5′ end of forward and reverse primers, respectively) and ligated into the pMW7 expression vector. All mutations had been verified by DNA sequencing, and, except for the desired base changes, all the sequences were identical to that of rat CACT cDNA (AC: NM_053965) [14]. The resulting plasmids were transformed into *E. coli* C0214. Bacterial overexpression, isolation of the inclusion body fraction, solubilisation and purification of the wild-type CACT and mutant CACT proteins were performed as described previously [10,19].

2.3. Reconstitution of wild-type and mutant CACT proteins in liposomes

The recombinant proteins were reconstituted into liposomes as described previously [10,19]. The concentration of intraliposomal carnitine was 15 mM.

2.4. Transport measurements

The external substrate was removed by passing 550 μl of proteoliposomes through a Sephadex G-75 column. The first 600 μl of the turbid eluate from the Sephadex column were collected, transferred to reaction vessels (100 μl each), and readily used for transport measurement by the inhibitor-stop method [21]. For uptake measurements, transport at 25 °C was started by adding 0.1 mM [^{3}H]carnitine (4.5 μCi/μmol) to proteoliposomes and at the required time interval the reaction was terminated by the addition of 1.5 mM NEM. In controls, the inhibitor was added together with the labelled substrate at time zero to evaluate the amount of labelled substrate unspecifically bound and/or diffused into liposomes. Finally, the external substrate was removed by chromatography on Sephadex G-75 columns, and the radioactivity in the liposomes was measured [21]. The experimental values were corrected by subtracting control values, which were always lower than 5% of the experimental values. All of the transport activities were determined by taking into account the efficiency of reconstitution (i.e., the share of successfully incorporated protein).

2.5. Other methods

SDS–PAGE was performed according to Laemmli [22] as previously described [10]. The amount of recombinant protein was estimated on Coomassie blue-stained SDS–PAGE gels by the Bio-Rad GS-700 Imaging Densitometer equipped with the software Bio-Rad Multi-Analyst, using bovine serum albumin as standard. The extent of incorporation of the recombinant protein in liposomes was determined as described in Phelps et al. [23], with the modifications reported in Ref. [19].

3. Results

3.1. Inhibition by H_2O_2 and activity recovery by DTE, NAC and Cys

The effect of 1 mM H_2O_2 on [^{3}H]carnitine/carnitine antiport has been studied adding the reagent to proteoliposomes 1 min before the labelled substrate (Fig. 1). The time course of the antiport was sensibly impaired by H_2O_2 (closed symbols) respect to the control (open symbols). Fitting the data points into first order rate equation, the initial rates of transport were derived as the product of the first order rate constant k and the transport at equilibrium. The values were 0.24 ± 0.03 nmol/min/mg protein for the control and 0.15 ± 0.02 nmol/min/mg protein for the inhibited sample. The treated samples showed, besides reduced transport rate respect to the control, also reduced transport at equilibrium, 2.30 ± 0.25 nmol/mg protein respect to 3.67 ± 0.32 nmol/mg protein of the control, indicating that a significant fraction of the protein was completely inactivated by H_2O_2. Indeed, under the conditions of reconstitution used, i.e., one protein molecule per proteoliposome, the transport at equilibrium is proportional to the amount of functional transporters [24]. Interestingly, DTE, a disulphide reducing agent, added after 20 min as well as after 60 or 120 min (small closed symbols), reverted the inhibition. DTE had a slight activation effect also on the control, without added H_2O_2 (▼), since a fraction of protein, which is spontaneously oxidised during the purification and reconstitution procedures, was