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Short communication

The mitochondrial oxidoreductase CHCHD4 is present in a semi-oxidized state in vivo

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ABSTRACT

Disulfide formation in the mitochondrial intermembrane space is an essential process catalyzed by a disulfide relay machinery. In mammalian cells, the key enzyme in this machinery is the oxidoreductase CHCHD4/Mia40. Here, we determined the in vivo CHCHD4 redox state, which is the major determinant of its cellular activity. We found that under basal conditions, endogenous CHCHD4 redox state in cultured cells and mouse tissues was predominantly oxidized, however, degrees of oxidation in different tissues varied from 70% to 90% oxidized. To test whether differences in the ratio between CHCHD4 and ALR might explain tissue-specific differences in the CHCHD4 redox state, we determined the molar ratio of both proteins in different mouse tissues. Surprisingly, ALR is superstoichiometric over CHCHD4 in most tissues. However, the levels of CHCHD4 and the ratio of ALR over CHCHD4 appear to correlate only weakly with the redox state, and although ALR is present in super-stoichiometric amounts, it does not lead to fully oxidized CHCHD4.

1. Introduction

The introduction of disulfide bonds in the mitochondrial intermembrane space (IMS) is an essential process [1–3]. A wide variety of processes depends on disulfide formation, including IMS protein import, Ca^{2+} signaling, relocalization of proteins to mitochondria during cellular stress, apoptosis, hypoxia signaling, tissue regeneration, and respiratory chain assembly [4–11]. Besides conserved pathways like IMS protein import, most of these processes are limited to higher eukaryotes and might even be tissue specific. However, most of our mechanistic knowledge on the mitochondrial disulfide relay is derived from work in *Saccharomyces cerevisiae* and there is only limited knowledge on the mammalian machinery for disulfide formation in the IMS.

A dedicated machinery, the mitochondrial disulfide relay, catalyzes IMS disulfide bond formation. This relay consists of the oxidoreductase CHCHD4 (in yeast: Mia40) and the sulfhydryl oxidase augmenter of liver regeneration ALR (also GFER, hsErv1, in yeast: Erv1) [4,5,12,13]. CHCHD4 contains a redox-active cysteine pair (CPC, where C represents cysteine and P proline), which in its oxidized state can introduce disulfide bonds into substrate proteins. Depletion of CHCHD4 results in strongly delayed oxidation kinetics of its substrates in intact cells [14]. Since oxidation is for many CHCHD4 substrates coupled to their IMS import [7,14], the levels of selected disulfide-containing IMS proteins decrease upon CHCHD4 depletion in human tissue culture cells [14–16]. After introducing disulfides into its substrates, CHCHD4 is present in its reduced state and requires ALR for reoxidation. As it is the case for CHCHD4, depletion of ALR delays substrate oxidation however to a lower extent [14]. From ALR electrons are shuttled via cytochrome *c* to complex IV [17,18]. CHCHD4 and ALR can in part replace their yeast counterparts, implying functional homology of the mammalian and yeast pathways at least for the processes that are conserved [19,20].

The redox state of the active site cysteines of an oxidoreductase is a

Abbreviations: DTT, dithiothreitol; GSH, reduced glutathione; IMS, intermembrane space of mitochondria; MTS, mitochondrial targeting sequence; mmPEG, methyl-polyethylene glycol-maleimide; NEM, N-ethylmaleimide; TCA, trichloroacetic acid; TCEP, Tris(2-carboxyethyl)phosphine

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fundamental indicator of its function. For CHCHD4 the redox states of its cysteines are not known. In yeast Mia40 the CPC motif is present in a partially reduced state (ca. 70–80% oxidized, 20–30% reduced) and this redox state is mainly controlled by Erv1 and the IMS glutathione pool [21]. The oxidized form of Mia40 thereby represents the form that mediates oxidative protein folding. Consequently, shifting the redox state of Mia40 to a more reduced state by either Erv1 depletion or glutaredoxin overexpression impairs oxidative protein folding [21,22]. Instead, the reduced portion of Mia40 could serve in principle in disulfide isomerization, which has so far only been demonstrated in vitro [23,24].

In the present study, we investigated the CHCHD4 redox state, and CHCHD4 and ALR amounts in cultured mammalian cells and mouse tissues. We thereby demonstrate that the redox state of the active site of CHCHD4 is partially reduced similarly to its yeast homolog. In vivo, the redox state of CHCHD4 but also the stoichiometry with its partner ALR differ between different tissues. Notably, although ALR is present in superstoichiometric ratios over CHCHD4 in almost all tissues, it does not seem to be sufficient to maintain CHCHD4 in a completely oxidized state implying diverse and partially independent functions of both proteins.

2. Results and discussion

2.1. The redox state of CHCHD4 is semioxidized in tissue culture cells

The major determinant for the proper function of CHCHD4 as an oxidoreductase is the redox state of its active site cysteines C53 and C55. The redox state of human CHCHD4 in intact cells and in vivo has not been investigated so far.

Human CHCHD4 exists in two isoforms which contain seven (isoform 1) or six cysteines (isoform 2), respectively (Fig. 1A and [9]). Four of these cysteines are in a so called structural twin CX_9C motif, while two cysteines are in the redox-active CPC motif (C53, C55) [25]. Isoform 1 contains an additional cysteine at position 4 (C4),while isoform 2 is slightly longer (155 aa vs 142 aa). Both isoforms are ubiquitously expressed on mRNA level in different tissues [9], and the presence of an additional cysteine in the N-terminal region of CHCHD4s of various species is conserved. In HEK293 cells, it appears that only isoform 1 is expressed on protein level, and we thus could limit our interpretations to this isoform with seven cysteines (Fig. S1).

In vitro, the reduction potential of the CHCHD4 redox-active CPC motif has been determined to be − 200 mV [25]. The reduction potentials of its structural disulfides are not known. However, the reduction potentials of the structural disulfides in other members of the twin CX_9C family of proteins are characterized by very low reduction potentials (< −300 mV) [26,27]. Thus, we expected that the twin CX_9C motif contains two stable, hard-to-reduce disulfide bonds, while the remaining cysteines in CHCHD4 should be easily reducible with minute amounts of reductants. To test this, we first investigated the accessibility of CHCHD4 cysteines to thiol-modifying agents upon treatment with reducing agents (Fig. 1A). To this end, we treated lysates of HEK293 cells with the reductant tris(2-carboxyethyl)phosphine (TCEP) at 4 °C and 96 °C and subsequently modified free (i.e. reduced) cysteines with the alkylating compound methyl-PEG-12-maleimide (mmPEG12) which covalently modifies free thiols and thereby slows the migration of modified proteins on SDS-PAGE. We thereby found that reduction at 4 °C only led to a small change in migration on SDS-PAGE compared to unmodified CHCHD4, which likely corresponds to modification of the reduced CPC motif, and C4 of CHCHD4. Conversely,

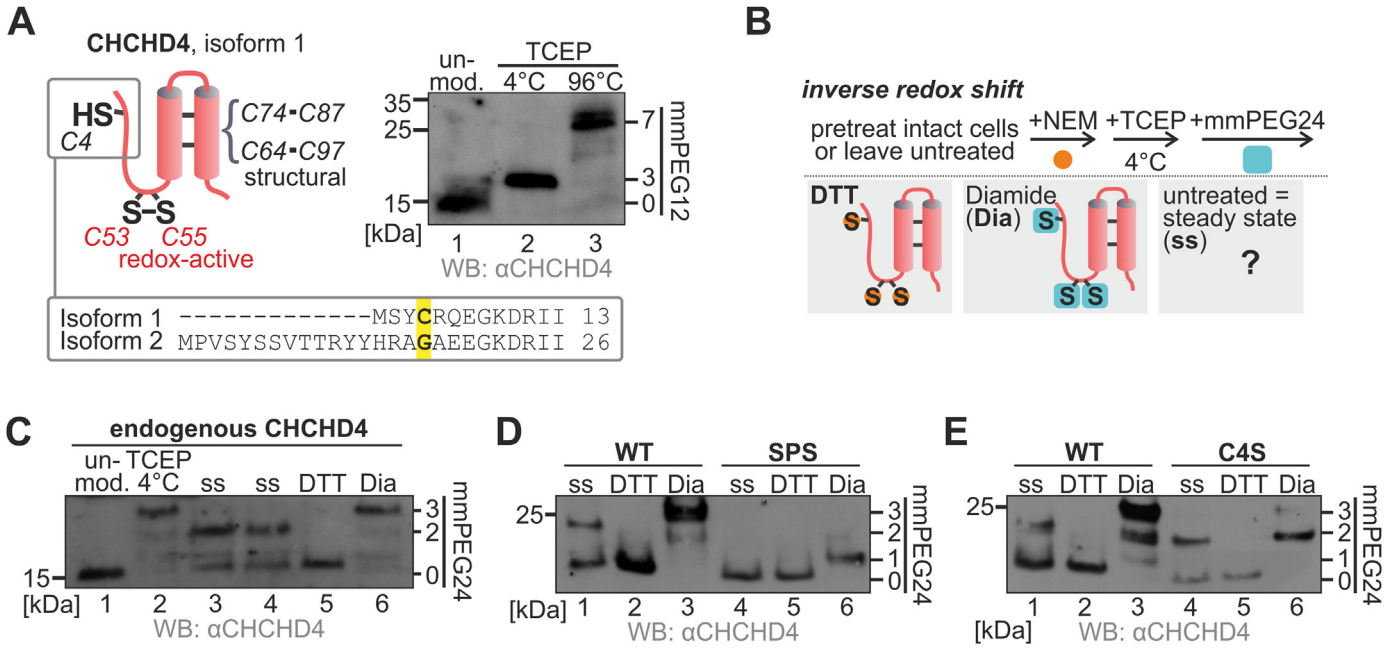

Fig. 1. The active-site cysteines in human CHCHD4 are mainly oxidized in intact cells. (A) *The layout of human CHCHD4.* Isoform 1 of CHCHD4 contains seven cysteines four of which are found in two disulfides in the structural CX_9C motif of CHCHD4. Two more are present in the redox-active CPC motif (C53/C55) and one additional is present at the N-terminus of CHCHD4 (C4, only in isoform 1). The structural disulfides in CHCHD4 are stable against treatment with the reductant TCEP at low temperature. To determine stability cell lysates were treated with TCEP at 4 °C or 96 °C and subsequently modified with mmPEG12. (B) *Layout of inverse redox state experiment.* To determine the redox state of CHCHD4 at steady state, intact cells were treated with NEM to stop all thiol-disulfide exchange reactions and trap the redox state of CHCHD4. Then, cells were lysed and lysates were treated at 4 °C with TCEP. Previously oxidized cysteines in the CPC motif are now accessible to modification with mmPEG24. As controls for the oxidized or reduced CPC motif, intact cells were treated with the oxidant diamide or the reductant DTT, respectively. As further controls, unmodified samples and samples without NEM pretreatment were loaded (not shown in the scheme). (C-E) *Redox state determination of endogenous CHCHD4 and CHCHD4 cysteine variants.* The experiment was performed as indicated in (B). HEK293 cells (C) and HEK293 cell lines stably expressing different variants of CHCHD4 (D, E) were used. The CPC motif of endogenous CHCHD4 is mainly oxidized, while C4 is reduced. Overexpression of CHCHD4 results in a slightly more reduced redox state and absence of C4 results in a more oxidized redox state of the CPC motif. The redox state experiment for endogenous CHCHD4 in HEK293 cells was performed 32 times (see Fig. 2D for quantification).