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Short communication

Oxidative folding and reductive activities of *Eh*PDI, a protein disulfide isomerase from *Entamoeba histolytica*

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ABSTRACT

PDI enzymes are oxidoreductases that catalyze oxidation, reduction and isomerization of disulfide bonds in polypeptide substrates. We have previously identified an *E. histolytica* PDI enzyme (*Eh*PDI) that exhibits oxidase activity *in vivo*. However, little is known about the specific role of its redox-related structural features on the enzymatic activity. Here, we have studied the *in vivo* oxidative folding of *Eh*PDI by mutagenic analysis and functional complementation assays as well as the *in vitro* oxidative folding and reductive activities by comparative kinetics using functional homologues in standard assays. We have found that the active-site cysteine residues of the functional domains (Trx-domains) are essential for catalysis of disulfide bond formation in polypeptides and proteins, such as the bacterial alkaline phosphatase. Furthermore, we have shown that the recombinant *Eh*PDI enzyme has some typical properties of PDI enzymes: oxidase and reductase activities. These activities were comparable to those observed for other functional equivalents, such as bovine PDI or bacterial thioredoxin, under the same experimental conditions. These findings will be helpful for further studies intended to understand the physiological role of *Eh*PDI.

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The correct formation of disulfide bonds between cysteine residues is an important biochemical modification of many proteins. Early observations showed that disulfide bond formation proceeds much faster *in vivo* than *in vitro*, suggesting the existence of a catalyst for protein oxidative folding in living cells [1]. The eukaryotic enzyme protein disulfide isomerase (PDI, EC 5.3.4.1), an oxidoreductase, catalyzes the oxidation, reduction, and isomerization of disulfide bonds in polypeptides [2]. PDI enzymes have functional Trx-domains (homologous to the prokaryotic enzyme thioredoxin) that contain the active-site motif CXXC, in which the cysteine residues cycle between dithiol and disulfide oxidation states [2,3]. Moreover, it has been demonstrated that these cysteine residues are essential for the enzymatic activity, since their biochemical modification causes irreversible inactivation [4]. Furthermore, substitutions of cysteine to serine or alanine residues have a major influence on the activity of PDI [5–7].

To study the functional activity of PDI enzymes *in vivo*, yeast and bacterial cells have been successfully used to complement phenotypes associated with defective disulfide bond formation [7,8]. In *Escherichia coli* cells, the isomerization and net formation of disulfide bonds takes place in the periplasmic compartment [9]. The major catalyst of net formation of disulfide bonds is DsbA [10,11], while the isomerization is catalyzed predominantly by the DsbC protein [12–14].

For *Entamoeba histolytica*, the causative agent of human amebiasis, the formation of accurate intra- or inter-molecular disulfide bonds is an important biochemical modification during protein folding, including some virulence factors [15,16]. Thus, the identification and characterization of enzymes that play key roles in protein oxidative folding is essential to understand this biochemical process. By performing a post-genomic analysis, we found that *E. histolytica* has a PDI family comprised of 11 polypeptides; moreover, the structure–function relationship predicted different oxidoreductase activities for some members of the family [17]. We have identified an entamoebal PDI enzyme (*Eh*PDI) that functionally complements the *dsb*A mutation in *E. coli* [18]. Here, we have studied the *in vivo* oxidative folding catalyzed by *Eh*PDI using mutagenic analysis and functional complementation assays, as well as the *in vitro* oxidative folding and reductive activities by comparative kinetics using functional homologues in standard assays.

The *in vivo* functional complementation assay was carried out using *E. coli* JCB571 strain (*dsb*A mutant). This strain constitutively expresses an alkaline phosphatase that is dependent on the oxidative folding activity of periplasmic DsbA [10]. *Eh*PDI, wild type or mutants, were expressed overnight in uninduced cultures and the bacterial periplasmic fraction was isolated by chloroform osmotic shock. The alkaline phosphatase activity was determined by using a previously published assay [8,18].

Abbreviations: PDI, protein disulfide isomerase; Trx, thioredoxin.

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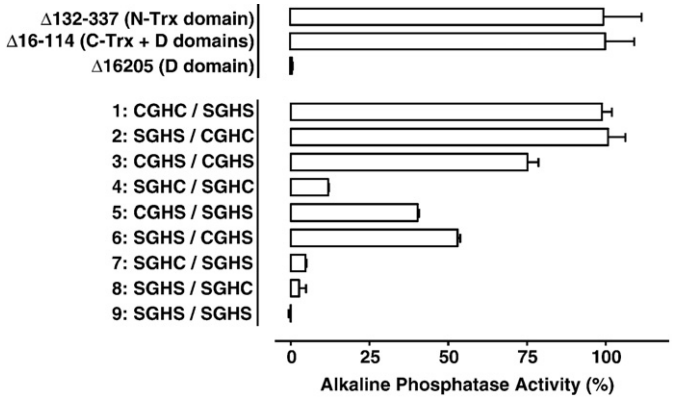

Fig. 1. *In vivo* oxidative folding activity of *Eh*PDI mutants. Results are represented as percentage of restoration of the periplasmic alkaline phosphatase (AP) activity. Mean and standard deviation of three independent experiments are shown. The experiments were normalized using the AP activity of JCB571 harboring pBPelB:*Eh*PDI or pBluescript SK − as 100 and 0%, respectively [18]. At the steady state, the alkaline phosphatase activity was defined as the change of OD at 415 nm per minute per mg of periplasmic proteins. Upper panel, domain deletion mutants; lower panel, site-directed mutants.

Since *Eh*PDI has two Trx-domains that exhibit the structural features of active domains [17], the initial approach was to experimentally prove that both of them have *in vivo* oxidative folding activity when expressed without the background activity of the other. The full recovery of alkaline phosphatase activity confirmed our prediction (Fig. 1, upper panel). Thereafter, each cysteine residue, from the active-site motif CGHC, was targeted for site-directed substitutions [19]. Three synthetic oligonucleotides were designed for mutagenic PCR: (i) N44/C160 (5′-ttt gct ccg tgg agt gga cat tg-3′), for substitutions C44S and C160S; and, (ii) N47 (5′-gtg gac att cga aaa aac ttg-3′) and (iii) C163 (5′-gtg gac att cga agg ctc tc-3′), for substitutions C47S and C163S, respectively. Mutation-specific endonuclease cleavage and automatic DNA sequencing was performed to check each *Eh*PDI variant and protein expression was analyzed by SDS-PAGE or immunoblotting (data not shown).

We have found that none of the single mutations or domain inactivation, by double substitutions (Fig. 1, Bars 1 and 2), had any significant effect on the oxidative folding activity of *Eh*PDI; furthermore, double mutations of both carboxy-cysteine residues have little effect (Fig. 1, Bar 3). However, a significant effect was observed when both amino-cysteine residues were substituted (Fig. 1, Bar 4). We further investigated the oxidative folding activity of *Eh*PDI harboring a single active-site cysteine residue, without any background activity from the other residues. *Eh*PDI mutants having any of the carboxy-cysteine residues retain 40 to 50% of the oxidative folding activity (Fig. 1, Bars 5 and 6), while mutants that harbor any of the amino-cysteine residues lost more than 95% of the activity (Fig. 1, Bars 7 and 8). Finally, and as expected, the oxidative folding activity of *Eh*PDI is fully dependent on the presence of the active-site cysteine residues, since the mutant with the four cysteine residue substitutions completely lost the enzymatic activity (Fig. 1, Bar 9).

Additionally, to test the oxidative folding and reductive activities in *vitro*, recombinant *Eh*PDI was expressed and purified. For this purpose, the cDNA encoding the mature polypeptide was amplified by PCR and subcloned in-frame into the pQE30 plasmid (Qiagen). Recombinant plasmid was transfected into *E. coli* XL1-Blue MRF' cells (Stratagene) and protein expression was performed according to standard protocols. Recombinant *Eh*PDI was isolated under denaturing conditions, refolded in-column and purified following protocols recommended by the Qiagen's QIA*expressionist*™ Handbook. A final desalting and exchange buffer procedure was carried out by gel filtration using a PD-10 column (GE Healthcare). As judged by SDS-PAGE analysis, the purity degree of the recombinant *Eh*PDI was acceptable (>95%).

The oxidative folding activity of *Eh*PDI was tested using denatured and reduced RNAse (Sigma) as substrate and following a standard method [20]. When recombinant *Eh*PDI was added into the reaction buffer, the rate of cCMP (Sigma) hydrolysis was increased compared to the uncatalyzed reaction, where a slow spontaneous renaturation was observed (Fig. 2A). The active RNase A concentration was calculated considering the competitive inhibition of CMP upon the hydrolysis of cCMP. In our experimental conditions, bovine PDI (Sigma) and *Eh*PDI enzymes exhibit comparable renaturation activities at the steady state (0.0392 ± 0.0004 and 0.0329 ± 0.0014 μmol of active RNase A per minute per μmol of PDI enzyme, respectively).

The reductive activity of *Eh*PDI was analyzed using bovine insulin (Sigma) as substrate in a turbidimetric assay [21]. The recombinant *Eh*PDI was enzymatically active, since the precipitation of the free B-chain of insulin occurred rapidly, compared with the reaction without PDI (Fig. 2B). However, at the steady state, the specific reductive activity was slightly lower (1.247 ± 0.029 units of OD_{650} per minute per mg of *Eh*PDI enzyme) compared with those observed for bovine

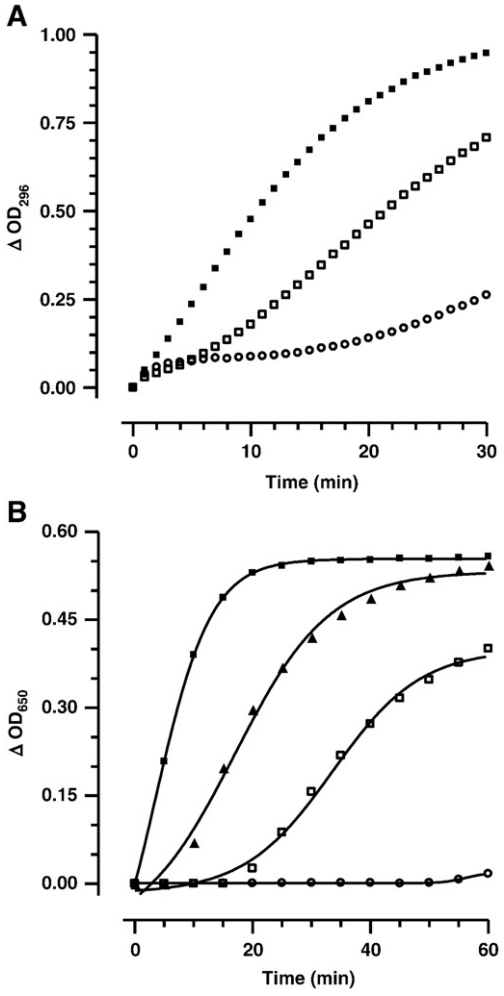

Fig. 2. *In vitro* oxidative folding and reductive activities of recombinant *Eh*PDI. (A) RNase A oxidative folding assisted by *Eh*PDI. Bovine PDI (closed square) or *Eh*PDI (open square) were added (4 μM, final concentration) to the reaction buffer. Reaction without PDI enzyme represents the spontaneous renaturation of the RNase A (open circle). Oxidative refolding was evaluated by measuring the OD at 296 nm. Each point is the mean of a duplicate experiment. (B) Thiol-disulfide reduction of bovine insulin by *Eh*PDI. Bovine PDI (closed square), bacterial Trx (closed triangle) or *Eh*PDI (open circles) were added (100 mg/mL, final concentration) to the reaction buffer. Reaction without thiol-reductive enzyme represents the spontaneous reduction of bovine insulin (open circle). The reduction was evaluated by measuring the OD at 650 nm. Each point is the mean of a triplicate experiment.

PDI and bacterial Trx (Sigma) (3.290 ± 0.361 and 1.991 ± 0.174 units of OD_{650} per minute per mg of thiol-reductase enzyme, respectively).

The availability of a simple *in vivo* assay to test the oxidative folding activity, plus the aid of homology-based prediction of structure–function relationships, and the mutagenic analysis or recombinant expression of other PDI enzymes with related activities were useful to study some functional roles of the *Eh*PDI enzyme. Here, we have demonstrated that the active-site cysteine residues of the Trx-domains are essential for catalysis of disulfide bond formation in polypeptides, such as the bacterial alkaline phosphatase. Moreover, we have showed that the recombinant *Eh*PDI enzyme has some typical properties of PDI enzymes: oxidase and reductase activities. Furthermore, these activities were comparable to those observed in other functional equivalents, such as the bovine PDI or the bacterial thioredoxin, under the same experimental conditions.

Although it must be demonstrated, *Eh*PDI represents a potential target for the development of alternative anti-amebic chemotherapeutics; this idea is supported by inhibition studies carried out with homologues from other protozoan parasites [22–24]. So, inhibition of enzymes involved in protein folding could deprive *E. histolytica* of functional and essential proteins for its parasitic life style. The detailed analysis of the structural features of the human and entamoebal PDI enzymes might be useful to design more specific and selective inhibitors. For instance, the domain organization of the human PDI (abb′a′c) and *Eh*PDI (aa′D) are different. Furthermore, several PDI-like proteins with different domain organization and, probably, physiological functions are known to co-exist with conventional PDI in yeast and mammals [25,26]. In *E. histolytica*, we have identified a PDI family of 11 members; six of them display the characteristic features of functional enzymes [17]. Among these, 206.m00090 (which corresponds to *Eh*PDI) is likely to be the major PDI enzyme involved in protein folding in *E. histolytica*, because it is the only one that closely resembles other homologue features (such as the two functionally active Trx-domains) and it is actively expressed in the trophozoite stage of the parasite [18]. Finally, in order for *Eh*PDI to be considered as a putative target for the development of new anti-amebic drugs based on an impaired folding of exported parasite proteins, knock down or silencing experiments will allow us to evaluate the essential role of this enzyme for the *E. histolytica* life style, including virulence and survival.

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