UNIVERSIDAD AUTÓNOMA DE BAJA CALIFORNIA

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Plegamiento oxidativo de proteínas en *Entamoeba histolytica*: La quitinasa amibiana (*Eh*CHT1) depende de un correcto plegamiento oxidativo. ¿Es factible silenciar a la oxidorreductasa amibiana *Eh*PDI?

TESIS

QUE PARA OBTENER EL GRADO DE
DOCTOR EN CIENCIAS

P R E S E N T A

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Tesis de Doctorado

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Abreviaturas

ABREVIATURA	SIGNIFICADO
°C	Grado centígrado
µm	Micrómetro
AG	Aparato de Golgi
ARNdc	Ácido ribonucleico de doble cadena
ARNi	Ácido ribonucleico de interferencia
ARNm	Ácido ribonucleico mensajero
ARNp	Ácido ribonucleico pequeño
ARNp-AS	Ácido ribonucleico pequeño anti-sentido
ARNpi	Ácido ribonucleico pequeño de interferencia
ARNta	Ácido ribonucleico de tallo y asa
ATF6	Factor de activador de la transcripción-6
ATP	Adenosín trifosfato
BiP	Proteína de unión a inmunoglobulina
CBD	Dominio de unión a quitina
CP	Cisteína proteasa
CRD	Dominio de reconocimiento de carbohidrato
DIC	Contraste de Interferencia Diferencial
EDEM	Chaperona tipo manosidasa que aumenta la degradación en el RE
*Eh*CHT1	Proteína quitinasa de *Entamoeba histolytica*
*Eh*CP	Cisteína proteasa de *Entamoeba histolytica*
*Eh*KERP	Proteína rica en lisina y ácido glutámico de *Entamoeba histolytica*
*Eh*P29	Peroxirredoxina de *Entamoeba histolytica*
*Eh*PDI	Proteína disulfuro isomerasa de *Entamoeba histolytica*
*Eh*STIRP	Proteína rica en serina, treonina e isoleucina de *Entamoeba histolytica*
ERAD	Degradación asociada al RE
Gal/NAcGal	Galactosa y N-acetilgalactosamina
GFP	Proteína verde fluorescente
GPI	Glicofosfatidil inositol
GuCl	Cloruro de Guanidinio
Hgl	Subunidad pesada de la lectina
Hsp	Proteína de choque térmico
Hsp70	Proteína de choque térmico de 70 kDa
Hsp90	Proteína de choque térmico de 90 kDa
Igl	Subunidad intermedia de la lectina
IRE1	Proteína que requiere inositol-1
kDa	Kilodalton
kJ	KiloJoule
Lgl	Subunidad ligera de la lectina
M	Molaridad
mg	Miligramo
min	Minuto
PDI	Proteína disulfuro isomerasa
PERK	Proteína cinasa de RE parecida a proteína cinasa activada por ARN
PPIasa	Peptidil-prolil *cis-trans* isomerasa
RE	Retículo endoplásmico
r*Eh*CHT1	Proteína quitinasa recombinante de *Entamoeba histolytica*
RT-qPCR	Retrotranscripción seguida de una reacción en cadena de la polimerasa cuantitativa.
T	Temperatura
Trx	Tiorredoxina
UF	Unidades de fluorescencia
UPR	Respuesta a proteínas no plegadas

Resumen

Entamoeba histolytica es el protozoario parasítico causante de la amibiasis en el humano. Aunque su distribución es cosmopolita, principalmente afecta a países en vías de desarrollo, donde se ubica dentro de las primeras causas de morbilidad. Su ciclo de vida alterna entre dos estadios estables: el quiste (forma infectiva) y el trofozoíto (forma invasiva).

El quiste brinda resistencia al parásito frente a condiciones medioambientales adversas. De manera interesante, este estadio se caracteriza por la presencia de una pared de quitina, la cual es sintetizada y degradada durante los procesos enquistamiento y desenquistamiento, los cuales son clave para completar el ciclo celular. Las quitinasas son glicosil hidrolasas que rompen los enlaces β(1,4)-glucosídicos entre los residuos de N-acetilglucosamina de la quitina. El genoma de *E. histolytica* codifica para una quitinasa amibiana (*Eh*CHT1) que se expresa de manera específica en la pared de quitina, sugiriendo su participación en los procesos de diferenciación celular.

En el presente proyecto se analizaron las características bioquímicas y biofísicas de la enzima *Eh*CHT1 recombinante. Los resultados mostraron que es una enzima estable en un amplio rango de pH y de temperatura, pero sensible a moderadas concentraciones de un agente químico desnaturalizante, sugiriendo que su función catalítica depende de una estructura correctamente plegada. Además, como su dominio catalítico contiene tres residuos de cisteína, de los cuales se predice la formación de un enlace disulfuro, se examinó la dependencia de la actividad catalítica al estado oxidativo de su estructura nativa. Los resultados de un estudio paralelo demostraron que la función catalítica de *Eh*CHT1 depende de un correcto plegamiento oxidativo, el cual puede ser asistido por una oxidorreductasa amibiana (*Eh*PDI).

El genoma de *E. histolytica* codifica para una familia de 11 proteínas PDI; de las cuales, *Eh*PDI es expresada activamente en el estadio de trofozoito. Mediante ensayos *in-vitro* e *in-vivo* (en modelos heterólogos), se determinó que exhibe las características estructurales y funcionales típicas de enzimas PDI; sin embargo, la elucidación de su papel funcional en la fisiología del parásito aún está pendiente.

Por tal motivo, se ha propuesto analizar la función de *Eh*PDI mediante una estrategia de silenciamiento génico, vía activación del mecanismo de *ARNi*. Como primera estrategia experimental, se ha planteado la técnica de alimentación amibiana con bacterias que expresan moléculas de *ARNdc* específicas. Los experimentos aún están en proceso; por lo tanto, no es posible adelantar resultados. En caso de que la tecnología aplicada no rinda resultados a corto plazo, se ha considerado aplicar otras estrategias metodológicas para establecer de manera inequívoca la función de *Eh*PDI en la fisiología y estilo de vida de *E. histolytica*. Más aún, determinar su potencial como diana terapéutica para el diseño de moléculas con propiedades farmacológicas anti-amibiasis.

Abstract

The protozoan parasite *Entamoeba histolytica* is the causal agent of human amebiasis. Although it has a worldwide distribution, it mainly affects developing countries, where it is positioned in the first causes of morbidity. The life cycle of this protozoan parasite is alternated between two stable stages: cyst (infective phase) and trophozoite (invasive phase).

The cyst gives the parasite the capacity to withstand harsh environmental conditions. Interestingly, this stage is characterized by a chitin wall that is synthesized and degraded during the encystation and excystation processes which have a key role to complete the cell cycle. Chitinases are glycosyl hydrolases enzymes that break β(1-4) glycosidic bonds in N-acetylglucosamine residues in chitin. The genome of *E. histolytica* codes for an amebic chitinase (*Eh*CHT1) that is specifically expressed in the chitin wall, suggesting its functional role in cellular differentiation processes.

In this research work, the biochemical and biophysical features of the recombinant enzyme r*Eh*CHT1 were analyzed. It was shown it is a stable enzyme in a wide range of pH and temperature, although it is sensitive to mild concentrations of a chemical denaturant agent, suggesting its activity depends on a correct protein folding. Furthermore, as its catalytic domain has three cysteine residues, which (according to *in-silico* prediction) are forming a disulfide bond, the dependence of the catalytic activity on the oxidative state of its native structure was analyzed. Results of a parallel analysis showed the catalytic function of *Eh*CHT1 depends on a correct oxidative folding that can be assisted by an amebic oxidoreductase (*Eh*PDI).

The genome of *Entamoeba histolytica* codes for a family of 11 PDI proteins; from which, *Eh*PDI is actively expressed in the trophozoite stage. By *in-vitro* and *in-vivo* (in heterologous models) assays, it was determined that *Eh*PDI shows the structural and functional features of typical PDI enzymes; although, the elucidation of its functional role in the physiology of the parasite is still pending.

For this reason, we propose to analyze *Eh*PDI function by a gene silencing strategy, activating the interference RNA mechanism. As a first experimental strategy, it is proposed to feed ameba with bacteria that expresses dsRNA specific molecules. Experimental assays are still in process, so we cannot anticipate any result. In case this technology does not give short term results, it has been considered to apply other strategies to unequivocally establish *Eh*PDI function in the physiology and the life style of *E. histolytica*. Moreover, to determine its potential as a therapeutic target to design molecules with pharmacological properties anti-amebiasis.

I. INTRODUCCIÓN

I.1. Amibiasis y *Entamoeba histolytica*

La amibiasis es la enfermedad infecciosa causada por el parásito protozoario llamado *Entamoeba histolytica*. Inicialmente, el parásito invade el intestino grueso, produciendo amibiasis intestinal; sin embargo, en ciertos casos migra hacia otros tejidos, principalmente el hígado, generando amibiasis extra-intestinal [Ximénez *et al.*, 2007]. A pesar de que *E. histolytica* es un parásito de distribución cosmopolita, los mayores índices de prevalencia de la enfermedad se han observado en países en vías de desarrollo, primordialmente aquellos de regiones tropicales y subtropicales donde la accesibilidad a servicios sanitarios es limitada [Ximénez *et al.*, 2007]. A nivel mundial, la amibiasis es la tercera causa de muerte por parasitosis [Sánchez y Mujica, 2009]. Pese a que las proyecciones estiman 500 millones de infectados, se ha reportado que sólo el 10% de esa población desarrolla la enfermedad; siendo la amibiasis intestinal la infección más frecuente (80-98%) [Araujo, *et al.*, 2008].

I.2. Amibiasis en México

La amibiasis continúa ubicándose dentro de las primeras veinte causas de morbilidad en la población mexicana, presentándose con mayor frecuencia (22.1%) en los estados de las regiones sur y sureste del país (Figura 1) [Sánchez y Mujica, 2009].

I.3. Ciclo de vida del parásito

El ciclo de vida de *E. histolytica* depende de dos estadios estables: el quiste (fase infectiva) y el trofozoíto (fase invasiva). La vía de transmisión es oral-fecal y la infección se adquiere por ingerir quistes maduros (tetra-nucleados) presentes en bebidas, alimentos,

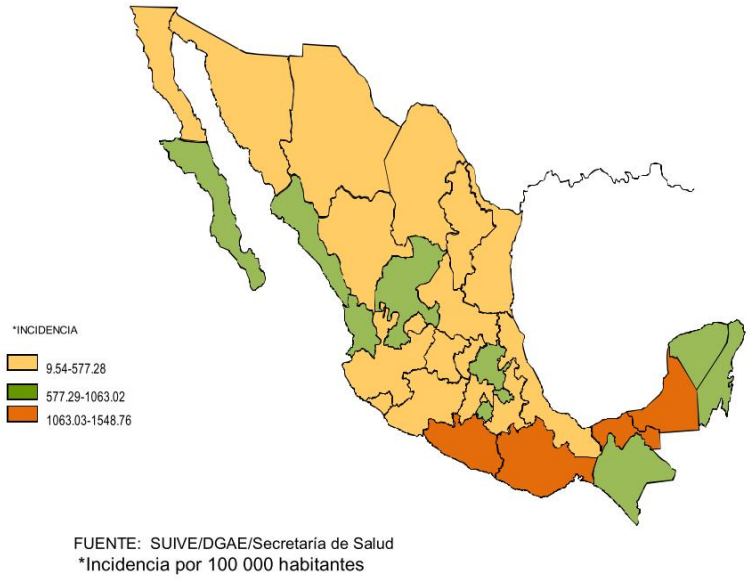

Figura 1. Incidencia de amibiasis intestinal en México (reporte 2008) [Sánchez y Mujica, 2009].

o manos contaminadas con heces. Cuando el quiste pasa por el estómago su pared se reblandece y al continuar por el intestino delgado sufre un proceso de desenquistamiento, liberándose un trofozoíto tetra-nucleado. En esta etapa, los núcleos sufren una división adicional, generando un trofozoíto octa-nucleado. Finalmente, éste sufre un proceso de fisión celular y produce ocho trofozoítos mono-nucleados. De manera global, cada quiste maduro ingerido originará ocho trofozoítos viables.

Habitualmente, los trofozoítos (20-40 µm de diámetro) colonizan el intestino grueso, causando la infección intestinal. Sin embargo, al ser la fase móvil del parásito, ocasionalmente migran hacia otros órganos, produciendo la enfermedad extra-intestinal. De manera alterna, el trofozoíto continúa por el tracto intestinal, produciendo una pared de quitina y generando un quiste mono-nucleado, que al madurar se convierte en un quiste tetra-nucleado (después de dos duplicaciones nucleares continuas). Los quistes maduros son liberados en heces, completando el ciclo (Figura 2) [Ximénez *et al*., 2011].

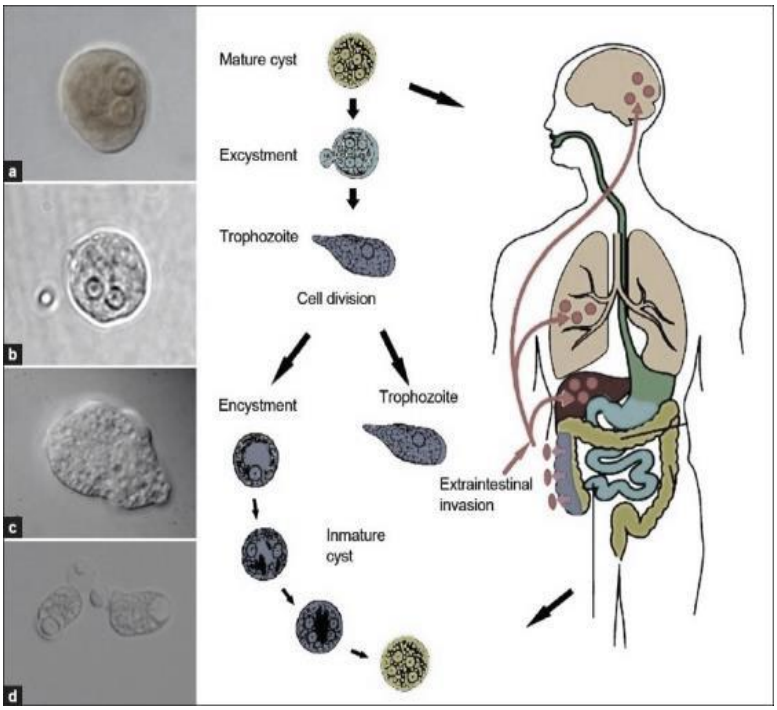

Figura 2. Ciclo de vida de *E. histolytica*.

(a) Quiste teñido con lugol al 4 % (100X). (b) Quiste sin teñir (100X). (c) Trofozoíto observado mediante contraste de interferencia diferencial (DIC) (100X). (d) Trofozoítos con eritrocitos fagocitados (DIC) (40X). Tomado y traducido de Ximénez (2011). El texto de la imagen permanece en el idioma original de referencia.

I.4. Patogenia molecular de la amibiasis

La patogenia de la amibiasis resulta de la interacción compleja de varios factores, tanto del parásito como del hospedero y del medioambiente. Específicamente, la virulencia de *E. histolytica* depende de su capacidad de secretar moléculas involucradas en diversos procesos patológicos, tales como adhesión y degradación de la mucosa intestinal, ruptura de la barrera epitelial, desregulación del transporte de iones, inflamación sistémica, absorción inadecuada de nutrientes, y alteración de la microbiota [Marie y Petri, 2014]. A continuación, de manera breve se describen los factores de virulencia más importantes involucrados en la patogenia de la amibiasis.

Lectina de unión a galactosa y N-acetilgalactosamina (*Gal/NAcGal*). Esta lectina es una de las principales moléculas de adhesión, uniéndose específicamente a residuos *Gal/NAcGal* presentes en glicoproteínas de membrana de la célula diana. El *core* de esta lectina es un heterodímero (260 kDa) que consta de las subunidades Hgl (pesada) y Lgl (ligera), unidas por enlaces disulfuro, el cual se asocia a la subunidad Igl (intermedia) mediante enlaces no covalentes [Barroso *et al*., 2014]. Hgl es una proteína transmembranal (170 kDa) que posee un dominio de reconocimiento de carbohidrato (CRD) rico en residuos de cisteína. Es la subunidad clave en la adherencia amibiana. Lgl es una proteína (31-35 kDa) anclada a la membrana mediante GPI (glicofosfatidilinositol). Igl es una proteína de reciente identificación y ha sido asociada a las actividades hemolíticas y citotóxicas del parásito [Barroso *et al*., 2014; Kato *et al*., 2015]. Para ejercer actividad lítica sobre el tejido, es necesaria la adhesión de *E. histolytica* a proteínas de la membrana celular y a componentes de la matriz extracelular; por tal motivo, esta lectina es considerada un factor de virulencia muy importante para las interacciones parásito-hospedero [Welter, *et al*., 2011].

Amibaporos. Estos son polipéptidos pequeños (77 residuos de aminoácidos) que conforman una familia de tres isoformas (A, B, y C). Poseen actividad citolítica sobre bacterias y células del hospedero mediante la inserción en membrana, generando pequeños poros y favoreciendo la permeabilización y lisis [Andrä *et al*., 2003; Ralston y Petri, 2011]. Se almacenan en gránulos citoplasmáticos y son secretadas después de la adhesión del parásito a la membrana de la célula diana. La isoforma A es la más abundante y de mayor importancia para la virulencia del parásito. Los estudios de silenciamiento génico (mediante ARN*i*) reportaron una disminución del 60% en su nivel de expresión y una drástica reducción en la patogenicidad amibiana [Bracha *et al*., 2003; Ralston y Petri, 2011].

Cisteína proteasas. Estas proteínas corresponden a una familia de aprox. 50 enzimas proteolíticas dependientes de cisteína, denominadas *Eh*CP. De manera interesante, se observó que las proteasas *Eh*CP1, *Eh*CP2, y *Eh*CP5 son responsables del 90% de la actividad CP total del parásito [Lee *et al*., 2015]. Las *Eh*CPs están involucradas en las

actividades citolítica y citotóxica de la amiba, ejerciendo un efecto citopático sobre tejido epitelial, causando daño a las neuronas entéricas, y confiriendo la capacidad de evadir el sistema inmune. Como sustratos fisiológicos (del hospedero) de estas proteasas amibianas se encuentran mucina, vilina, laminina, y colágeno; las anafilotoxinas C3a y C5a; interleucina 18 y las inmunoglobulinas IgA e IgG; el proteoglicano, y diferentes componentes de la matriz extracelular, entre otros [Lindell *et al*., 2006; Ralston y Petri, 2011; Lee *et al*., 2015].

Peroxirredoxina (*EhP29*). Durante el proceso invasivo, el parásito se enfrenta a un estrés oxidativo generado por el sistema de defensa celular del hospedero. Habitualmente, las células eucarióticas poseen diversos mecanismos de respuesta a ese tipo de estrés (por ejemplo, superóxido dismutasa, glutatión peroxidasa, catalasa, el sistema de glutationes), los cuales juegan un papel importante en la sobrevivencia celular [Sen *et al*., 2007]. Aunque *E. histolytica* carece de catalasa y el sistema de glutationes, posee una peroxirredoxina dependiente de tiol de 29 kDa (*Eh*P29) que se sobre-expresa durante el estrés oxidativo [Akbar *et al*., 2004; Sen *et al*., 2007]. *Eh*P29 se asocia a la lectina de unión a *Gal/NAcGal* durante la interacción parásito-hospedero, sirviendo como un mecanismo de defensa contra el ataque oxidativo activado por el hospedero, facilitando la invasión tisular [Sen *et al*., 2007].

EhSTIRP. Este factor es una proteína rica en serina, treonina e isoleucina (*Eh*STIRP) que se expresa de forma abundante en cepas virulentas de *E. histolytica*. Mediante experimentos de silenciamiento génico, se demostró que participa en procesos amibianos importantes, como adhesión y citotoxicidad [MacFarlane and Singh, 2007].

EhKERP. Es otro factor importante para el proceso infeccioso, llamado KERP1 por su alto contenido en lisina y ácido glutámico (*Lysine and Glutamic Rich Protein* 1) [Perdomo *et al*., 2013]. Se almacena en vesículas y puede localizarse tanto en membrana del trofozoíto como en el espacio intersticial ubicado entre el parásito y la célula diana [Seigneur *et al*., 2005]. Por su capacidad de unirse a la superficie de células epiteliales del intestino y por sus altos niveles de expresión en cepas virulentas, ha sido involucrada en el

proceso de adhesión amibiana. Además, su silenciamiento génico produce una reducción en la capacidad amibiana de formar abscesos hepáticos (en un modelo animal) [Santi-Rocca *et al*., 2007; Perdomo *et al*., 2013].

II. ANTECEDENTES Y GENERALIDADES

II.1. La Quitinasa amibiana (*Eh*CHT1)

II.1.1. Quitinasa

Las quitinasas son glicosil hidrolasas que rompen el enlace β(1-4) entre los residuos de N-acetilglucosamina que conforman al polisacárido quitina. Genes codificantes para quitinasas se han identificado en una amplia gama de organismos (bacterias, hongos, nemátodos, insectos, plantas, y mamíferos [de la Vega *et al.*, 1997; Ruíz-Sánchez *et al.*, 2005]. Actualmente, se han dilucidado diferentes moléculas con potencial inhibidor de la actividad quitinolítica; sin embargo, la más reconocido es un antibiótico producido por la bacteria *Streptomyces sp.*: alosamidina, que es un inhibidor competitivo específico de las quitinasas [Hamid R. *et al*., 2013].

II.1.2. Quitinasas de *E. invadens*

Para transitar entre los diferentes estadios de su ciclo de vida, *E. histolytica* depende de dos procesos importantes de diferenciación celular: enquistamiento y desenquistamiento. A la fecha, no ha sido posible completar el ciclo en condiciones *in vitro*. Afortunadamente, para este fin, se ha utilizado a *Entamoeba invadens* (causante de amibiasis en reptiles) como modelo heterólogo. El genoma de *E. invadens* codifica para cuatro quitinasas: *Ei*CHT1, *Ei*CHT2, *Ei*CHT3, y *Ei*CHT4, las cuales se expresan de manera diferencial durante su ciclo de vida, indicando que la actividad quitinolítica es una función importante para la diferenciación celular (enquistamiento ↔ desenquistamiento) [Muñoz *et al.*, 2016].

II.1.3. Quitinasa de *E. histolytica*

Contrariamente, *E. histolytica* codifica sólo una quitinasa activa: *Eh*CHT1, la cual exhibe una identidad cercana al 70% con respecto a las quitinasas de *E. invadens*. *Eh*CHT1 ha sido identificada como una proteína específica de la pared quística, sugiriendo que juega un papel clave en los procesos de enquistamiento y desenquistamiento amibiano [de la Vega *et al.*, 1997; Muñoz *et al.*, 2016].

De manera interesante, se ha demostrado que alosamidina retrasa el enquistamiento de *E. invadens* en cultivos axénicos [Hamid R. *et al*., 2013]. Con este antecedente, y debido a la alta homología entre las quitinasas amibianas, es factible suponer que la inhibición de *Eh*CHT1 puede arrestar el ciclo de vida de *E. histolytica* y, por lo tanto, detener la infección. Sin embargo, para considerar a *Eh*CHT1 como diana terapéutica para el diseño de nuevos agentes anti-amibianos, es esencial comprender sus propiedades bioquímicas únicas y reconocer sus particularidades estructurales y funcionales como objetivos estratégicos [Muñoz *et al.*, 2016].

II.2. La vía de secreción de proteínas

II.2.1. Transporte y secreción de proteínas

En células eucarióticas, la vía de secreción de proteínas está conformada por el retículo endoplásmico (RE), el aparato de Golgi (AG), las vesículas de transporte, lisosomas, y membrana celular [Farhan y Rabouille, 2011]. Esta vía es responsable del transporte y entrega de proteínas desde el RE hasta su destino final, ya sea el medio extracelular, la membrana plasmática, o algún compartimento endomembranoso [Farhan y Rabouille, 2011; Muñiz y Riezman, 2015].

En el RE, se llevan funciones altamente especializadas, como la síntesis y plegamiento correcto de proteínas, y la síntesis de lípidos. Además, ese compartimento

juega un papel importante en el mantenimiento de la homeostasis intracelular de calcio [Chang *et al*., 2015]. Por su parte, el AG participa en las modificaciones post-traduccionales de proteínas y en la clasificación de moléculas de custodia (cargo), que serán enviadas hacia sus respectivos destinos finales [Álvarez-Miranda *et al*., 2015; Machamer, 2015]. Por lo tanto, ambos compartimentos juegan un papel importante en la proteostasis celular.

II.2.2. Chaperonas y plegasas

La proteostasis celular se refiere a todos aquellos procesos celulares que mantienen el equilibrio entre la síntesis, plegamiento, desplegamiento, y degradación de proteínas. Siendo las biomoléculas funcionalmente más importantes, existen diversos factores que afectan su estabilidad, por ejemplo, la secuencia de aminoácidos, el nivel de expresión, la actividad específica, las modificaciones post-traduccionales, y su asociación con otras biomoléculas [Mares *et al*., 2015; Muñoz *et al*., 2015].

Ante lo anterior, es necesario que la célula cuente con un mecanismo altamente regulado que le permita mantener una homeostasis adecuada. Para tal fin, emplea una maquinaria de control de calidad, participando en una red de proteostasis y protegiendo a las proteínas de la siguiente manera: (i) evitando la adopción de un amplio margen de conformaciones estructurales, especialmente en un ambiente tan conglomerado como el intracelular, (ii) actuando como puntos de verificación de plegamiento, y (iii) regulando su síntesis, recambio, y tráfico intracelular. De manera importante, un desequilibrio en la proteostasis puede conducir a la acumulación de polipéptidos mal plegados, o desplegados, promoviendo la formación de agregados, resultando en proteotoxicidad y estrés, por último, muerte celular [Mares *et al*., 2015; Muñoz *et al*., 2015; Radwan *et al*., 2017].

Las chaperonas y plegasas son proteínas que responden a condiciones de estrés, asistiendo el plegamiento y replegamiento de polipéptidos. Debido a la importancia de su función, se encuentran conservadas a lo largo de la escala evolutiva, desde células procariotas hasta organismos eucariotas superiores [Muñoz *et al*., 2015]. La interacción

transitoria con polipéptidos no plegados o mal plegados evita la agregación proteica en diferentes compartimentos sub-celulares, tales como RE, mitocondria, núcleo, y citoplasma [Vega *et al*., 2016].

BiP (*Hsp70*). Las chaperonas más destacas corresponden a la familia de proteínas de choque térmico (Hsp, *Heat shock protein*), clasificadas por su talla molecular en: 100, 90, 70, 60, 40, y 27). Cada clase de Hsp presenta funciones especializadas, pero no separadas, ya que actúan en conjunto en los mecanismos de respuesta al estrés térmico, potenciando la renaturalización proteica, restaurando la proteostasis, y promoviendo la sobrevivencia de la célula [Lackie *et al*., 2017]. La chaperona BiP (*Immunoglobulin binding protein*) es una Hsp70 que participa en el plegamiento asistido en el RE, uniéndose a sustratos y estabilizando el estado no plegado de los mismos. Su función depende de ciclos de hidrólisis del ATP, propiciando la unión y liberación del sustrato hasta obtener la estructura nativa [Ramírez *et al*., 2017]. Además, participa en la translocación de polipéptidos nacientes hacia el RE, dirige a las proteínas no plegadas correctamente hacia un proceso de degradación especializado (ERAD, *ER-associated degradation*), y regula la homeostasis de calcio. De manera importante, BiP participa como co-sensor del estrés del RE [Wang *et al*., 2009]. La acumulación de proteínas no plegadas conduce a un estrés en el RE, promoviendo la activación de la vía UPR (*Unfolded protein response*). Esta activación es iniciada por tres proteínas transmembranales que actúan como sensores: IRE1 (*Inositol requiring enzyme* 1), PERK (*PKR-like ER kinase*), y ATF6 (*Activating transcription factor* 6). En condiciones fisiológicas estables, los tres sensores se encuentran inactivos mediante la interacción de sus dominios luminales con BiP. Alternativamente, en condiciones de estrés en el RE (específicamente, por acumulación de proteínas no plegadas), BiP se disocia, lo cual favorece la activación de las vías de respuesta mediadas por los sensores (IRE1, PERK, y ATF6). El objetivo de UPR es reestablecer la homeostasis del RE, en caso contrario, promoverá la muerte celular [Wang *et al*., 2009; Oslowski y Urano, 2011].

Calnexina y calreticulina. Son lectinas que actúan como chaperonas durante el plegamiento de glicoproteínas, formando parte de la maquinaria de control de calidad,

evitando que proteínas mal plegadas abandonen el RE [Wang *et al*., 2017]. En este compartimento, después de la N-glicosilación, las glucosidasas I y II remueven dos residuos de glucosa, generando una glicoproteína mono-glucosilada. En ese momento, calnexina y calreticulina interactúan con la glicoproteína para favorecer la adopción de la estructura conformacional nativa. De manera casi inmediata, la plegasa ERp57 (tiol oxidasa) se une al complejo lectina-glicoproteína y cataliza la formación de los enlaces disulfuro requeridos en la glicoproteína (para estabilizar su estructura). Posteriormente, la glucosidasa II remueve el último residuo de glucosa, propiciando la desagregación del complejo y liberación de la glicoproteína [Ellgaard y Helenius 2003; Wang *et al*., 2017]. Si la glicoproteína adquirió su estructura nativa, puede abandonar el RE. En caso contrario, será mono-glucosilada nuevamente, por acción de una glucosiltransferasa, interaccionará con las lectinas y será sometida a rondas de plegamiento asistido. Sin embargo, si la glicoproteína presenta continuamente un plegamiento incorrecto, será reconocida como sustrato para la α-manosidasa I, que remueve un residuo de manosa, y se promoverá su degradación vía EDEM (*ER degradation-enhancing 1,2-mannosidase-like protein*) y ERAD [Ellgaard y Helenius 2003; Roth y Zuber 2017].

Peptidil-prolil cis-trans isomerasa (PPIasa). Esta plegasa pertenece a una superfamilia de enzimas ubicuas que catalizan la isomerización cis-trans de residuos de prolina presentes en polipéptidos en proceso de plegamiento [Dunyak y Gestwicki, 2016]. El mecanismo de isomerización aún no está completamente elucidado; sin embargo, a diferencia de otras chaperonas o plegasas, las enzimas PPIasa no hidrolizan ATP. Además, su catálisis no involucra la ruptura o formación de ningún tipo de enlace, realiza una rotación alrededor del enlace peptidil-prolil, favoreciendo así una conformación adecuada para el plegamiento correcto [Quistgaard *et al*., 2016].

Proteína disulfuro isomerasa (PDI). En mamíferos, esta plegasa pertenece a una gran familia de 21 integrantes. Se localiza principalmente el RE, aunque puede fugarse hacia otros compartimentos de la vía de secreción, incluyendo la membrana citoplasmática y el espacio extracelular. Como plegasa, cataliza la reducción, oxidación, e isomerización

de enlaces disulfuro; además, exhibe funciones como chaperona, asistiendo el plegamiento oxidativo de proteínas [Mares *et al*, 2015; Bekendam *et al*., 2016].

II.2.3. Participación de PDI en el plegamiento de proteínas

Principalmente en el RE, las plegasas PDI participan asistiendo el plegamiento oxidativo y el control de calidad de polipéptidos nacientes. Sin embargo, aunque no se ha determinado claramente cómo escapan de ese compartimento, también han sido localizadas en la membrana citoplasmática de células de mamíferos, tales como linfocitos, plaquetas, células endoteliales, hepatocitos, y en una amplia diversidad de células cancerosas. De manera interesante, la PDI de membrana exhibe actividad reductasa sobre proteínas de superficie celular, aumentando la adhesión celular [Pan *et al*., 2014; Bekendam *et al*., 2016].

Las plegasas PDI son proteínas multifuncionales que varían en longitud y arreglo de dominios. Sin embargo, como característica estructural en común poseen al menos un dominio funcional parecido a la tiorredoxina (*Trx*) bacteriana, el cual exhibe la estructura secundaria típica βαβαβαββα, y contiene el motivo catalítico CXXC [Kozlov *et al*., 2010]. La PDI de mamífero, también conocida como PDIA1/P4HB, es la plegasa más estudiada; por lo tanto, es considerada como canónica. Típicamente, presenta la secuencia de dominios *a-b-b´-x-a´*, donde *a* y *a´* son dominios *Trx* funcionales, ambos conteniendo CGHC como motivo catalítico; en tanto, *b* y *b´* son dominios *Trx* no funcionales. De manera interesante, ambos dominios *b* son ricos en residuos hidrofóbicos, siendo importantes para la unión al sustrato y la actividad chaperona de PDI. Además, *b´* y *a´* se encuentran unidos por un péptido flexible de 19 aminoácidos (llamado conector *x*). La estructura cristalográfica desplegó un arreglo tridimensional parecido a una herradura, donde *a* y *a´* conforman los brazos, uno frente al otro, en tanto *b* y *b´* la hendidura. Además, posee una secuencia señal en el N- terminal y el tetra-péptido KDEL en el C-terminal, que participan en su localización y retención en el RE, respectivamente [Kozlov *et al*., 2010; Mares *et al*., 2015].

Durante las primeras etapas de la síntesis de una proteína, pueden formarse enlaces disulfuro no nativos que impiden su plegamiento correcto. En este caso, PDI en su estado reducido ataca al enlace disulfuro incorrecto en la proteína-sustrato, iniciando un rearreglo de tal enlace, el cual puede ocurrir mediante dos vías: (i) un rearreglo intramolecular, o (ii) un ciclo de reducción-oxidación (Figura 4). En la primera vía (también denominada ciclo de isomerización), la proteína-sustrato juega un papel importante, ya que una vez que PDI atacó al enlace incorrecto, un residuo de cisteína de la proteína-sustrato ataca y desplaza a la PDI del complejo PDI-sustrato, favoreciendo el rearreglo sus enlaces disulfuro y liberando a PDI en su forma reducida. En la segunda vía, el residuo de cisteína libre, presente en el sitio catalítico involucrado en el ataque inicial, ataca y desplaza a la proteína-sustrato del complejo PDI-sustrato, liberando a la proteína-sustrato en su forma reducida y dejando a la PDI en su estado oxidado. Enseguida, otra PDI en su estado oxidado catalizará la formación del enlace disulfuro en la proteína-sustrato liberada [Wilkinson y Gilbert, 2004].

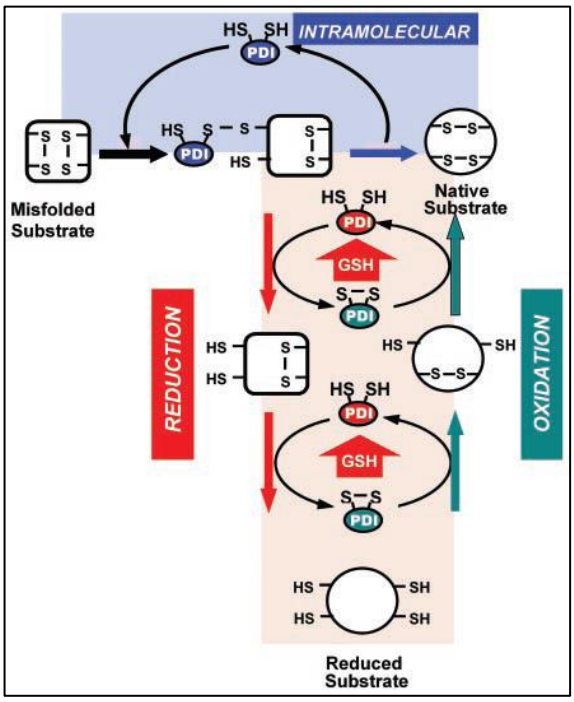

Figura 3. Mecanismo de la isomerización de enlaces disulfuro catalizada por PDI.

Tomado de Wilkinson y Gilbert (2004). El texto permanece en el idioma original de la referencia.

II.2.4. La proteína PDI implicada en diversas patologías

Típicamente, un desequilibrio en la proteostasis conduce a la acumulación de proteínas mal plegadas en el RE, produciendo un estado de estrés y activando el mecanismo de UPR. En caso de incompetencia celular para reestablecer el equilibrio, el sistema biológico promueve la muerte celular. En su carácter de plegasa multifuncional, la PDI apoya al mantenimiento de la proteostasis celular, jugando un papel clave tanto en el plegamiento como en la degradación de proteínas [Parakh y Atkin, 2015].

Recientemente, se han reportado evidencias que involucran a la PDI en diversas enfermedades en humanos. Por ejemplo, su sobre-expresión en algunos tipos de cáncer (próstata, ovario, y pulmón) y en algunas enfermedades neurodegenerativas (Alzheimer, esclerosis lateral amiotrófica, y Creutzfeldt-Jakob). Por otro lado, su expresión en superficie de plaquetas y células endoteliales ha sido involucrada en la formación inicial de trombos. Además, su expresión en membrana de linfocitos ha sido asociada al proceso de entrada del VIH, promoviendo la internalización de la partícula viral [Reiser *et al*., 2012, Mares *et al*., 2015; Parakh y Atkin, 2015]. Por lo anterior, ha ganado interés como potencial diana terapéutica [Mares *et al*., 2015].

II.2.5. Vía de secreción de *E. histolytica*

En eucariotas, el sistema endomembranoso involucrado en la vía de secreción de proteínas se encuentra conservado a lo largo de la escala evolutiva. De manera interesante, se ha reportado que *E. histolytica* carece de un sistema endomembranoso típico (RE y AG). En un estudio reciente, se mostraron evidencias de la continuidad del RE amibiano mediante co-localización de BiP y GFP (etiquetada con FLAG y KDEL) dentro de un compartimento endomembranoso [Teixeira y Huston, 2008]. Además de BiP, otros marcadores de la vía de secreción amibiana han sido identificados: Sec61, PDI, ERD2, calreticulina, NSF, COPI, COPII, SNAP, SNARE, Rab, y Vps [Perdomo *et al*., 2014]. Aún más, mediante un análisis de proteómica celular, se detalló un mapa de los componentes involucrados en el tráfico vesicular, que incluye cerca de 1500 proteínas. De manera

sorprendente, 67% de esos componentes corresponden a marcadores del RE y AG [Perdomo *et al.*, 2014]. Por lo tanto, aun careciendo de un sistema endomembranoso típico, *E. histolytica* posee una maquinaria molecular capaz de asistir el plegamiento, transporte, y secreción de proteínas.

II.3. Silenciamiento de *Eh*PDI

II.3.1. Oxidorreductasa amibiana *Eh*PDI

En células eucariotas, una gran cantidad de proteínas que transitan por la vía de secreción poseen enlaces disulfuro que son esenciales para una estructura y función adecuada. *E. histolytica* no es la excepción, pues una amplia diversidad de proteínas, incluyendo factores de virulencia, contienen enlaces disulfuro que estabilizan su estructura terciaria. En comparación con otros organismos, poco se conoce acerca de la maquinaria que cataliza el plegamiento oxidativo de proteínas amibianas; inclusive, el primer gen amibiano codificante para una PDI funcionalmente activa, denominada *Eh*PDI, fue reportado a principios de este siglo [Ramos y Alagón, 2000].

Actualmente, se conoce que el genoma de *E. histolytica* codifica para una familia de once proteínas PDI [Ramos *et al*., 2008]; de las cuales, la proteína *Eh*PDI ha sido estudiada y caracterizada ampliamente: es un polipéptido de 337 residuos, posee una secuencia señal N-terminal de 15 residuos, contiene dos dominios *Trx* funcionales (*a*: 16-120; *a´*: 131-235), ambos con el motivo catalítico CGHC y el patrón de plegamiento βαβαβαββα, y un dominio D, que pudiera estar implicado en un mecanismo evolutivamente divergente para la retención en el RE, tal como se reportó para su proteína homóloga en *Dictyostelium discoideum* (*Dd*PDI) [Ramos *et al* 2005; Ramos *et al*., 2008].

Además de las características estructurales, *Eh*PDI exhibe las actividades de una PDI típica: oxidasa, reductasa, e isomerasa. Una de las primeras evidencias fue la complementación funcional de la actividad oxidasa de la proteína DsbA de *E. coli*,

utilizando el periplasma bacteriano de una mutante Δ*dsb*A como plataforma celular para reestablecer la actividad catalítica de una fosfatasa alcalina dependiente de DsbA (como fenotipo cuantitativo) [Ramos *et al.*, 2005; Mares *et al.*, 2009]. Por otro lado, mediante estudios *in-vitro*, se demostró que posee las tres actividades típicas de una oxidorreductasa funcional: oxidasa, isomerasa, y reductasa, ya que fue capaz de catalizar eficientemente la oxidación e isomerización de lisozima y la reducción de insulina [Mares *et al*., 2009; Ramos *et al*., 2011]. De manera sorprendente, también se observó que funciona como chaperona, lo cual fue demostrado mediante ensayos *in-vitro* de: (i) prevención de la agregación de la cadena B de insulina (inducida por DTT), y (ii) protección contra la inactivación térmica de enzimas termosensibles (α-glucosidasa y NdeI) [Ramos *et al*., 2011; Mares *et al*., 2015].

Como puede notarse, la función de *Eh*PDI en su ambiente fisiológico no ha sido evaluada. Desafortunadamente, las características genéticas del parásito imposibilitan la utilización de técnicas típicas para la generación de mutantes, como recombinación homóloga. Por tal motivo, se han empleado diferentes técnicas de silenciamiento génico, donde figura la activación del mecanismo de ARN interferente (ARNi), como sistemas genéticos para el estudio de la función fisiológica de proteínas en *E. histolytica* [Solis *et al*., 2009].

II.3.2. Mecanismo de ARNi en *Caenorhabditis elegans*

ARNi es un mecanismo que emplean las células de manera natural para reducir la expresión de genes específicos. Este mecanismo fue descrito por Andrew Fire y Craig Mello, en 1998, utilizando al nemátodo *C. elegans* como modelo. Ellos determinaron que la introducción de *ARN* de doble cadena (*ARNdc*) producía la degradación del *ARNm* complementario al *ARNdc*. Una vez en el citosol, el *ARNdc* es reconocido e hidrolizado por *Dicer*, una enzima con actividad RNasa III, generando pequeños *ARNi* (de 20 a 25 nt de longitud), los cuales poseen 2 nt de cadena sencilla en el extremo 3´ y un grupo monofosfato en el extremo 5´. Los pequeños *ARNi* son cargados en un complejo denominado *RISC* (complejo de silenciamiento por ARN de interferencia), donde la

proteína *Ago* (Argonauta) reconoce e hidroliza a la hebra pasajera (no complementaria), mientras que la hebra de guía (complementaria) funcionará como sonda para el silenciamiento, ya que hibrida con el *ARNm* y promueve su degradación (vía *Ago*) [Zhang *et al*., 2011; Hoh, 2014].

II.3.3. ARNi en *E. histolytica*

Durante los últimos años, de manera alternativa a los procedimientos de genética clásica, se han utilizado diferentes técnicas para evaluar la función génica mediante silenciamiento génico (vía *ARNi*) [Bracha *et al*., 2003; Vayssiéa *et al*., 2004; MacFarlane y Singh, 2007]. El mecanismo *ARNi* en *E. histolytica* aún no ha sido dilucidado. Sin embargo, ya se han identificado genes amibianos que codifican para proteínas homólogas. Por ejemplo, se han identificado tres genes *Ago* (EHI_125650, EHI_186850, y EHI_177170) que contienen los dominios PAZ y PIWI. Es interesante que, *Ago2-2* (EHI_125650) presenta elevada expresión y ha demostrado capacidad de asociación con moléculas de ARN pequeñas (27 nt), sugiriendo su participación en el mecanismo *ARNi* amibiano. También se ha reportado un gen *RdRP* (EHI_139420), que codifica para una ARN polimerasa dependiente de ARN. En *C. elegans*, la proteína *RdRP* se asocia con secuencias de ARN pequeñas que poseen un extremo 5´-polifosfato e induce el silenciamiento génico de *ARNm* que contienen secuencias complementarias. Finalmente, aunque no se ha identificado un homólogo amibiano parecido a *Dicer*, contiene un gen que codifica para una RNasa III activa, la cual ha mostrado funcionalidad en el mecanismo de *ARNi* mediante experimentos de silenciamiento génico en la levadura *S. cerevisiae* [Zhang *et al*., 2011; Pompey *et al*., 2015].

A la luz de lo anterior, es factible presumir que *E. histolytica* posee un mecanismo parecido a *ARNi* (el cual puede ser inducido por una molécula de *ARNdc*) que permite tanto el silenciamiento de *Eh*PDI como la evaluación de su función en el estadio de trofozoito del parásito. Aún más, es posible suponer que el silenciamiento de un gen codificante para una proteína clave del proceso de plegamiento, tal como *Eh*PDI, puede resultar en la

pérdida de la función de proteínas importantes para la biología celular y de diversos factores de virulencia indispensables para su estilo de vida parasítico.

III. RESULTADOS Y DISCUSIONES

III.1. Papel de *Eh*CHT1 y *Eh*PDI en la biología de *Entamoeba histolytica*

Las proteínas pueden realizar su función de forma apropiada en virtud de poseer una estructura tridimensional correcta; es decir, una conformación nativa. Estas biomoléculas pueden adoptar diferentes conformaciones estructurales dependiendo de las condiciones prevalentes en el medio que las rodea (por ejemplo, pH, fuerza iónica, o temperatura); sin embargo, cada proteína demanda condiciones medioambientales específicas que favorecen su estructura nativa [England y Haran, 2011].

La quitinasa amibiana (*Eh*CHT1) juega un papel importante en el ciclo de vida del parásito, siendo clave en los procesos de diferenciación celular: enquistamiento y desenquistamiento. A pesar de su relevancia fisiológica, el conocimiento de su relación estructura-función era limitado. En consecuencia, se propuso realizar un estudio con enfoques bioquímicos y biofísicos para establecer el efecto del pH, la temperatura, y la concentración de cloruro de guanidinio (como desnaturalizante químico) sobre la actividad y la estabilidad de la enzima recombinante.

Inicialmente, la enzima recombinante (r*Eh*CHT1) fue obtenida en su forma pura y activa (1574 ± 22 UF min^{-1} mg^{-1}) mediante protocolos estándar de expresión heteróloga en el periplasma de *E. coli* y purificación bioquímica a partir de extractos bacterianos.

Tal como se esperaba (por su correspondencia a una hidrolasa ácida), r*Eh*CHT1 exhibió una actividad catalítica mayor al 50% en el intervalo de pH = 3.2 - 6.3, con un valor óptimo estimado a 4.7. Además, conservó su estabilidad estructural (mayor a 90%) en el intervalo de pH = 5.0 - 8.5. No obstante, mostró inhibición e inestabilidad enzimática a pH extremos (menores a 3 y mayores a 10), lo que sugiere: (i) alteración en la carga neta de la proteína, vía protonación/desprotonación de residuos, (ii) desnaturalización de la estructura terciaria y, por ende, (iii) pérdida de la función.

De manera sorprendente, r*Eh*CHT1 mostró actividad catalítica en un amplio intervalo de temperatura, presentando actividad enzimática mayor a 50% en el intervalo de T = 40 - 60 °C y una temperatura óptima a 50 °C. También mostró absoluta termoestabilidad a valores de T ≤ 37 ºC; sin embargo, cuando ese umbral es rebasado con valores crecientes de temperatura, la actividad catalítica decae proporcionalmente (indicativo del proceso de desnaturalización proteica), observándose una pérdida total de la función a valores de T > 50 ºC. Adicionalmente, los análisis termodinámicos (considerando una cinética de inactivación de primer orden) revelaron que la enzima exhibe un tiempo de vida media de 73 horas a 37 ºC (temperatura corporal del hospedero), el cual aumenta hasta 3 años a 25 ºC (temperatura media fuera del hospedero).

Resulta interesante que la r*Eh*CHT1 mostró una notoria sensibilidad al cloruro de guanidinio (*GuCl*; agente desnaturalizante químico), observándose reducciones del 50% en su actividad catalítica y en su estabilidad proteica a concentraciones de cloruro de guanidinio de 0.88 y 0.50 M, respectivamente. Más aún, el valor estimado de energía libre (22.5 ±1.8 kJ mol^{-1} M^{-1}) sugiere una dependencia de la estabilidad de la proteína con la concentración de GuCl. Por lo tanto, es evidente que la presencia del agente desnaturalizante perturba la estructura terciaria de la proteína y, por ende, el sitio catalítico, inhibiendo la función.

Hasta este punto, los resultados obtenidos destacan la capacidad de *Eh*CHT1 para tolerar cambios rudos y súbitos de las condiciones medioambientales: pH y temperatura, prevalentes dentro y fuera del hospedero. Independiente a las implicaciones fisiológicas que representan para el estilo de vida de *E. histolytica*, la resistencia a un amplio intervalo de pH y su evidente termoestabilidad son características bioquímicas apreciables para fines biotecnológicos. Por otro lado, la desestabilización conformacional inducida por *GuCl* supone una debilidad estructural que conduce a una pérdida de la función. Además, es posible inferir que estos factores también pueden afectar procesos celulares esenciales, tales como plegamiento, desnaturalización, degradación, modificación, o procesamiento de proteínas [Deller *et al*., 2016].

Como característica bioquímica importante para su estructura terciara, diversas proteínas contienen enlaces disulfuro que estabilizan su conformación nativa y que, a su vez, son esenciales para su actividad funcional [Zhang *et al*., 2011]. Las quitinasas contienen un dominio de unión a quitina (denominado CBD) estabilizado por 3-4 enlaces disulfuro [Hamid *et al*., 2013]. *Eh*CHT1 no es excepción, pues posee 8 residuos de cisteína en su CBD altamente conservados y presumiblemente involucrados en la formación de 4 enlaces disulfuro. Por otro lado, la predicción tridimensional de su dominio catalítico muestra un plegamiento típico, similar al observado en dominios funcionales: un barril $(alfa/beta)_8$ tipo TIM; además, aparentemente, dos residuos de cisteína (Cys^{167} y Cys^{421}) forman un enlace disulfuro.

En un estudio paralelo, la dependencia de *Eh*CHT1 a un plegamiento oxidativo correcto fue examinada mediante ensayos de expresión funcional *in-vivo*, usando el periplasma bacteriano como un sistema heterólogo. Los resultados demostraron que su función catalítica depende tanto de un ambiente oxidante como de una plegasa con actividad disulfuro oxidasa. Congruente con lo esperado, esta observación confirma que cuando menos un enlace disulfuro es esencial para su estabilidad y función.

Más aún, los ensayos de complementación funcional revelaron que la plegasa *Eh*PDI es capaz de asistir el plegamiento oxidativo de *Eh*CHT1, demostrando, por primera vez, que presenta actividad catalítica (oxidasa) sobre sustratos fisiológicos. Por lo tanto, es factible suponer que participa en la formación de enlaces disulfuro de proteínas que transitan por la vía de secreción amibiana. Diversos factores de virulencia poseen enlaces disulfuro esenciales para su relación estructura-función, tales como la lectina de unión a Gal/NAcGal, las cisteína proteasas, y los amibaporos [Leippe *et al*., 1994; Jacobs *et al*., 1998; Ankri *et al*., 1999].

A la luz de lo anterior, es plausible considerar que *Eh*PDI participa en la formación de enlaces disulfuro durante el plegamiento de algunos factores de virulencia. Esta noción es apoyada por estudios realizados en diversos protozoarios, como *Plasmodium*, *Tripanosoma*, *Leishmania*, *Toxoplasma*, y *Giardia*, donde se ha reportado una clara

dependencia de los mecanismos que promueven un plegamiento eficiente y la actividad de los factores de virulencia exportados [Muñoz *et al*., 2015]. Con este antecedente, es factible suponer que la inhibición de plegasas esenciales puede conducir a una reducción o pérdida de virulencia del parásito y, por ende, controlar la infección.

III.2. Silenciamiento de *Eh*PDI mediante ARN interferente

Aunque no se ha dilucidado completamente el mecanismo de ARN de interferencia (*ARNi*) en *E. histolytica*, diversos genes amibianos han sido exitosamente silenciados usando diferentes técnicas que dependen de la maquinaria necesaria para degradar *ARNm*. A la fecha, entre las variantes tecnológicas que han comprobado su factibilidad experimental se encuentran: silenciamiento por (i) *ARNdc*, (ii) *ARNta*, y (iii) *ARNpi*, entre otros [Zhang *et al*., 2011; Khalil *et al*., 2016]. Mediante la expresión episomal de *ARNdc* se han estudiado los genes *Eh*Diaphanous, *Eh*KIp5, *Eh*KIP2-4, y *Eh*STIRP. De manera similar, *ARNta* ha sido útil para los genes *Eh*PATMK, *Eh*Igl-1, *Eh*URE-BP, *Eh*C2A, y *Eh*CPADH. Por otro lado, usando la técnica de embebido en *ARNpi* o *ARNdc* se han examinado los genes *Eh*KERP1, *Eh*(γ)-tubulina, y *Eh*Gln6Pi. La alimentación amibiana con bacterias que expresan *ARNdc*, una estrategia poco explorada, ha sido implementada para silenciar los genes *Eh*KERP1 y *Eh*(β)-tubulina [Zhang *et al*., 2011; Aguilar-Díaz *et al*., 2013; Ócadiz-Ruiz *et al*., 2016].

Una técnica que está captando la atención es el mecanismo alterno de silenciamiento génico basado en moléculas de tipo *ARNp* anti-sentido (*ARNp-AS*) [Morf *et al*., 2013]. De manera endógena, *E. histolytica* expresa una amplia variedad de moléculas de ARN de 27 nt (*ARNp*), los cuales poseen extremos 5´-polifosfato y son capaces de asociarse con *Eh*Ago2-2. Sorprendentemente, se observó que los niveles de expresión de *ARNp* correlacionan inversamente con la expresión del *ARNm* para el cuál son complementarios (p. ej., EHI_197520), sugiriendo su participación en la regulación de la expresión génica. Mediante el análisis de secuencias *ARNp-AS* dirigidas contra EHI_197520, se observó que son complementarios a una pequeña porción de la región

codificante, que fue denominada "región activadora". Aún más, se demostró que un segmento de tal región (132 pb) es suficiente para activar el mecanismo de silenciamiento y que la fusión con cualquier gen diana es suficiente para inducirlo. Esta tecnología fue satisfactoriamente caracterizada usando a la luciferasa como gen reportero; además, fue posible validarla utilizando los genes *Eh*ROM1 y *Eh*Myb. También, mediante un estudio de mejoramiento técnico, se demostró que la fusión de un segmento corto de la región codificante para el gen diana es suficiente para inducir el mecanismo celular [Khalil *et al*., 2016]. Además, se reportó que la técnica puede ser aplicada para silenciar dos genes (p. ej., *Eh*ROM1 y *Eh*Myb), de manera concomitante, cuando se fusionan con la región activadora.

Para establecer el papel funcional de *Eh*PDI en la fisiología de *E. histolytica*, como primera estrategia experimental se propuso el silenciamiento génico, vía activación del mecanismo de *ARNi*, usando la técnica de alimentación con bacterias que expresen una molécula de *ARNdc* gen-específica. Este método ofrece la ventaja de ser rápido y, además, con potencial para regular la expresión de genes amibianos [Solis *et al*., 2009]. Originalmente, la propuesta experimental fue planteada en cuatro etapas, descritas a continuación:

(1) Construcción de tres vectores bacterianos para la expresión de moléculas de *ARNdc*: dos dirigidos contra el gen *Eh*PDI (específicos para las secuencias 5´-promotora y codificante para el péptido maduro) y uno contra la secuencia codificante para GFP (control negativo).

(2) Transfección bacteriana, inducción y expresión de las moléculas *ARNdc*: La cepa *E. coli* HT115 (DE3), carente de la actividad RNAsa III (para evitar la degradación del *ARNdc*) y portadora de los vectores de expresión de las moléculas de *ARNdc*, se cultiva e induce en condiciones estándar de expresión.

(3) Alimentación de trofozoitos amibianos con bacterias que expresan *ARNdc*: las bacterias se suplementan a un cultivo amibiano en crecimiento exponencial, a una proporción de 10^4 bacterias por trofozoito, y la alimentación procede durante 48 h en condiciones típicas de cultivo.

(4) Evaluación del fenotipo molecular y celular resultante del silenciamiento génico de *Eh*PDI: el fenotipo molecular es analizado mediante ensayos de RT-qPCR e inmunoreconocimiento; en tanto, el fenotipo celular es determinado mediante ensayos de evaluación de capacidades (secretora y endocítica), así como el efecto citopático sobre un monocapa epitelial.

Actualmente, la propuesta se encuentra en estado pausado, habiéndose completado satisfactoriamente las dos primeras etapas. Los plásmidos pL5´Pro*Eh*PDI (que contiene una secuencia de 356 pb correspondiente al extremo 5´ y región promotora de *Eh*PDI), pLPM*Eh*PDI (que contiene la secuencia codificante para el péptido maduro de *Eh*PDI), y pLGFP (que contiene la secuencia codificante para GFP), fueron obtenidos mediante clonación molecular, usando plásmido pLitmus28i (New England Biolabs) como parental. Las diferentes moléculas de *ARNdc* fueron expresadas e inducidas adecuadamente en células de *E. coli* HT115 (DE3), portadoras de los respectivos plásmidos, siguiendo el protocolo recomendado por el proveedor.

De manera preliminar, la tercera y cuarte etapa han sido exploradas, observándose resultados prometedores, pero no concluyentes. Específicamente, se ha logrado normalizar el ensayo de alimentación y estandarizar el protocolo de evaluación del fenotipo molecular. Sin embargo, aún quedan pendientes los análisis de repetibilidad y reproducibilidad experimental.

En apariencia, esta propuesta es viable para determinar la participación de *Eh*PDI en la virulencia de *E. histolytica*; y por lo tanto, comprobar su potencial como posible diana terapéutica para el diseño racional de nuevos fármacos anti-amibianos, dirigidos para controlar y detener la infección parasitaria en humanos. En caso contrario, se ha considerado cumplir la meta usando alguna de las tres estrategias metodológicas siguientes: (1) cultivo embebido con *ARNdc* obtenido de bacterias, (2) cultivo embebido con *ARNpi* sintético, y (3) expresión episomal de *ARNp-AS*.

III.3. Vía de secreción de proteínas y la maquinaria del plegamiento como diana terapéutica

En cualquier célula, la maquinaria de plegamiento de proteínas es un componente indispensable para asegurar su supervivencia, ya que la fisiología celular depende de la función eficiente de miles de proteínas que realizan una amplia variedad de procesos biológicos complejos.

De manera similar, los protozoarios parasíticos cuentan con un mecanismo que promueve el plegamiento correcto de sus proteínas; especialmente, para aquellas que participan en procesos esenciales, como adquisición de nutrientes, liberación de factores de virulencia, adquisición de resistencia a fármacos, y evasión del sistema inmune de su hospedero, entre otros. Además, durante las diferentes etapas de su ciclo biológico, p. ej., infección e invasión del hospedero, estos se exponen a condiciones medioambientales adversas, como cambios bruscos de temperatura y de pH, que pueden afectar la estabilidad proteica. Por lo tanto, ¿cómo responder a condiciones de estrés y lograr completar su ciclo biológico? Específicamente, una maquinaria molecular, compuesta por chaperonas y plegasas, asiste a las proteínas para resistir las condiciones medioambientales adversas.

Ante esto, es factible proponer a componentes clave de la maquinaria de plegamiento de proteínas como dianas terapéuticas para el desarrollo de nuevos fármacos anti-parasitarios. Actualmente, la proteína Hsp90 de diversos parásitos ha sido utilizada como diana para el diseño de moléculas inhibidoras; sin embargo, la aplicación clínica de tales moléculas es incierta, ya que presentan una elevada toxicidad para el hospedero.

Por su parte, *E. histolytica* también posee componentes clave en la maquinaria de plegamiento proteínas, incluyendo una familia PDI. De manera interesante, *Eh*PDI se expresa de manera activa en el trofozoíto, sugiriendo que su participación es clave en ese estadio del ciclo biológico. Además, asiste el plegamiento oxidativo de otros factores esenciales para la diferenciación celular, p. ej., la quitinasa (*Eh*CHT1). Por tales motivos, es posible suponer que *Eh*PDI representa un blanco potencial para el diseño de nuevos

agentes anti-amibianos. Sin embargo, para corroborar la noción anterior, es necesario demostrar su papel funcional en la fisiología amibiana.

IV. CONCLUSIONES

La quitinasa amibiana (*Eh*CHT1) es estable en un amplio intervalo de pH y temperatura; sin embargo, su estructura funcionalmente activa se desestabiliza a moderadas concentraciones de cloruro de guanidinio (un agente desnaturalizante químico). Además, como su estructura terciaria posee enlaces disulfuro importantes para su actividad, depende de un plegamiento oxidativo correcto, el cual puede ser asistido por una oxidorreductasa amibiana (*Eh*PDI). Asimismo, como las quitinasas de *E. invadens*, un modelo heterólogo, juegan un papel importante en los procesos de diferenciación celular (enquistamiento/desenquistamiento), es factible suponer que la enzima *Eh*CHT1 representa un blanco potencial para diseño de fármacos dirigidos a detener el ciclo biológico de *E. histolytica* y, por ende, controlar la infección.

Actualmente, las opciones terapéuticas para el tratamiento de la amibiasis son limitadas; por tal razón, es necesario identificar proteínas esenciales que puedan ser utilizadas como blancos potenciales para el diseño de nuevos agentes terapéuticos. Los componentes de la maquinaria molecular (chaperonas y plegasas) que asiste al plegamiento de proteínas juegan papeles clave en diversos procesos celulares, incluyendo la capacidad de resistir condiciones medioambientales estresantes. Aunque *Eh*PDI ha mostrado actividad, tanto *in-vitro* como *in-vivo* (en modelos heterólogos), es imperativo realizar un análisis funcional en su ambiente fisiológico nativo. Para este fin, se propuso una estrategia metodológica de silenciamiento génico, basado en la activación del mecanismo de *ARNi*, mediante la técnica de alimentación amibiana con bacterias que expresan moléculas de *ARNdc* específicas.

El método de silenciamiento génico seleccionado es rápido y ha demostrado su potencial en el control de la expresión de genes amibianos. Sin embargo, los incidentes metodológicos que se presentaron durante el desarrollo experimental suponen la presencia de conflictos técnicos no sospechados, los cuales se asocian a la naturaleza del modelo.

Ante esto, ya se han identificado variantes metodológicas que aseguran una evaluación inequívoca el papel funcional de *Eh*PDI en la fisiología de *E, histolytica*.

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VI. ANEXOS

VI.1. Artículo: Muñoz *et al.* ACTIVITY, STABILITY AND FOLDING ANALYSIS OF THE CHITINASE FROM *Entamoeba histolytica*. Parasitology International 2016; 65: 70-77

Resumen

La amibiasis en humanos, causada por el protozoario parasítico *Entamoeba histolytica*, continúa siendo un problema importante de salud pública en países en vías de desarrollo. El ciclo de vida del parásito comprende dos etapas principales, el trofozoíto y el quiste, los cuales están ligados a dos eventos importantes: el enquistamiento y el desenquistamiento. De manera interesante, el quiste posee una pared de quitina que ayuda al parásito a soportar condiciones medioambientales adversas.

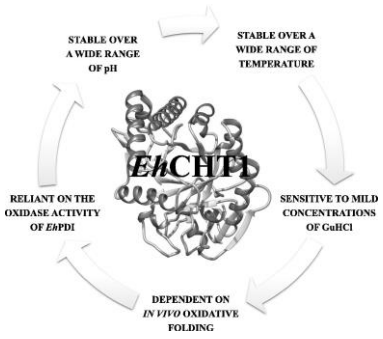

Figura 4. Resumen gráfico.

Tomado de Muñoz *et al*. (2016). El texto permanece en el idioma original de la referencia.

Debido a que la quitinasa amibiana ha sido reconocida como una molécula clave en ambos procesos, enquistamiento y desenquistamiento, es factible considerar que la inhibición específica de la enzima pudiera arrestar el ciclo de vida parasitario y, por tanto, detener la infección. Sin embargo, para inhibir de manera específica a *Eh*CHT1 es importante reconocer sus características bioquímicas únicas, lo que nos proporcionará herramientas para tener la habilidad de controlar su función celular. Por tanto, para reconocer la relación estructura-función de *Eh*CHT1, realizamos una serie de experimentos que nos permitieron examinar los efectos del pH, temperatura y de un agente desnaturalizante, sobre la actividad y estabilidad enzimática. Adicionalmente, la dependencia del plegamiento oxidativo *in-vivo* fue estudiada utilizando un modelo bacteriano. Nuestros resultados comprueban el potencial de *Eh*CHT1 como un blanco para el diseño y desarrollo de nuevos o mejorados agentes terapéuticos anti-amibianos. Así mismo, el potencial de la oxidorreductasa *Eh*PDI, involucrado en el plegamiento oxidativo de proteínas amibianas, también fue confirmado.

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Activity, stability and folding analysis of the chitinase from *Entamoeba histolytica*

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ABSTRACT

Human amebiasis, caused by the parasitic protozoan *Entamoeba histolytica*, remains as a significant public health issue in developing countries. The life cycle of the parasite compromises two main stages, trophozoite and cyst, linked by two major events: encystation and excystation. Interestingly, the cyst stage has a chitin wall that helps the parasite to withstand harsh environmental conditions. Since the amebic chitinase, *Eh*CHT1, has been recognized as a key player in both encystation and excystation, it is plausible to consider that specific inhibition could arrest the life cycle of the parasite and, thus, stop the infection. However, to selectively target *Eh*CHT1 it is important to recognize its unique biochemical features to have the ability to control its cellular function. Hence, to gain further insights into the structure–function relationship, we conducted an experimental approach to examine the effects of pH, temperature, and denaturant concentration on the enzymatic activity and protein stability. Additionally, dependence on *in vivo* oxidative folding was further studied using a bacterial model. Our results attest the potential of *Eh*CHT1 as a target for the design and development of new or improved anti-amebic therapeutics. Likewise, the potential of the oxidoreductase *Eh*PDI, involved in oxidative folding of amebic proteins, was also confirmed.

1. Introduction

Human amebiasis, the parasitic infection caused by the protozoan *Entamoeba histolytica*, remains as a considerable public health issue in developing countries [1]. The life cycle of *E. histolytica* includes two primary stages: trophozoite (invasive) and cyst (infectious). The trophozoite is able to colonize the large bowel, causing intestinal damage. While the cyst is resistant to adverse conditions outside the host and is highly contagious, being responsible for dissemination of the disease [2]. To cycle between stages, *E. histolytica* depends on two major processes: encystation and excystation [3,4]. Interestingly, the cyst has a chitin wall [5], which is thought help this cell stage to withstand harsh environmental conditions [6].

E. histolytica encodes one active chitinase, *Eh*CHT1 (GenBank U78319), that has been expressed as recombinant enzyme [7] and been identified as a cyst wall-specific protein [8]. Since completion of the life cycle of *E. histolytica* has not been possible *in vitro*, *Entamoeba invadens* (the reptilian counterpart) has been used as the model for study *Entamoebae* encystation and excystation processes [9]. *E. invadens* encodes four active chitinases, *Ei*CHT1, *Ei*CHT2, *Ei*CHT3, and *Ei*CHT4 (GenBank U78320, DQ324647, DQ324648, and AB576188, respectively), that are differentially expressed during its life cycle, indicating that the chitinase activity has a functional compromise in both processes [10,11]. As *Eh*CHT1 is highly similar to *E. invadens* homologs, and considering that allosamidin (a well-known chitinases inhibitor) can delay encystment when added to axenic cultures, it is plausible to suppose that inhibition of *Eh*CHT1 could arrest the life cycle of *E. histolytica* [4,7,9–11] and, thus, stop infection.

Recently, different approaches have been aimed toward discovering novel anti-amebic therapeutics [12–18]. However, targeting key proteins engaged in specific cellular processes remains as a feasible approach [19–22]. To have the ability of targeting *Eh*CHT1 and, thus, control its functional role in the life cycle of *E. histolytica*, it is essential to consider its unique biochemical features. Hence, to gain further insights into the structural and functional properties, we performed an experimental approach to examine the effects of pH, temperature, and guanidine hydrochloride on the activity and stability of the recombinant amebic chitinase (r*Eh*CHT1). Moreover, its dependence on oxidative folding was further studied by an *in vivo* model. Altogether, our results attest the potential of *Eh*CHT1 as a target for the design of specific anti-amebic therapeutics. Likewise, the potential of the oxidoreductase *Eh*PDI (GenBank AY730725), involved in oxidative folding of amebic proteins, was also confirmed.

2. Materials and methods

2.1. Materials

DNA amplification reagents and DNA purification kits were obtained from Qiagen. Bacterial culture media components were purchased from Becton Dickinson. Electrophoresis reagents were bought from Bio-Rad.

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Endonucleases and other enzymes were supplied by New England Biolabs. Other biochemicals were provided from Sigma. All materials were analytical or molecular biology grade reagents.

2.2. Bacterial strains, plasmids and growth conditions

Escherichia coli strains and plasmids used throughout this study are listed in Table 1. Bacterial cultures were grown in LB medium at 37 °C with constant agitation. When needed, appropriate concentration of antibiotics was supplemented to medium (ampicillin at 0.15 mg/mL; kanamycin at 0.025 mg/mL; chloramphenicol at 0.015 mg/mL). For *in vivo* oxidative folding assay, marker-free mutant Δ*dsb*A (MRB10) and Δ*dsb*C (MRB20) strains were obtained from the corresponding strain by excising the gene encoding for kanamycin resistance applying the FLP/frt recombination method [23,24]. Recombinant plasmids were constructed using standard molecular cloning protocols.

The plasmid used for expression and purification of r*Eh*CHT1 (pET22/*Eh*CHITv32) was obtained by cloning the sequence encoding for mature polypeptide (His^{16} to Cys^{507}) into the expression vector pET22b(+).The *Eh*Cht1 gene fragment was amplified from genomic DNA of *E. histolytica* (HK-9 strain) using a set of synthetic primers, 5′-cgg atc cca caa ctg tga agg tct ttc-3′ and 5′-ggt gga ctc gag aca ttt ctc aat tag act ct taa tgt-3′, designed to incorporate the BamHI and XhoI sites at the 5′- and 3′-end (underlined), respectively. The PCR product was digested with BamHI and XhoI endonucleases, and then ligated into the same sites of pET22b(+). Additionally, the BamHI site was engineered (cleaved–filled–autoligated) to get in-frame fusion with the PelB signal peptide codons. The gene authenticity and coding sequence fusions were confirmed by DNA sequencing: *Eh*CHT1 contains the N-terminal PelB leader and a C-terminal hexahistidine tag.

The plasmid used for *in vivo* oxidative folding of *Eh*CHT1 (pBAD-PelB-*Eh*CHIT84) was obtained by cloning the PelB-*Eh*Cht1 cassette into the expression vector pBAD33 [25]. The PelB-*Eh*Cht1 nucleotide sequence was amplified from pET22/*Eh*CHITv32 with the T7 universal primer, 5′-cgg atc cca caa ctg tga agg tct ttc-3′, and a synthetic primer, 5′-gaa gct ttt aac att tct caa tta gac-3′, designed to incorporate the HindIII site at the 3′-end (underlined). The PCR product was digested with XbaI and HindIII, and then ligated into the same sites of pBAD33. The authenticity of the inserted fragment was confirmed by stringent endonucleolytic analysis and DNA sequencing.

2.3. Expression and purification of recombinant EhCHT1

The r*Eh*CHT1 was expressed in the periplasmic compartment of *E. coli* cells and purified from bacterial lysates by standard procedures. Fresh subcultures (1:100) of *E. coli* BL21(DE3) cells harboring the plasmid pET22/*Eh*CHITv32 were grown in medium supplemented with ampicillin. After 2 h, the protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and cells were grown for an additional 4 h. The bacterial cells (from a 100-mL culture) were harvested by centrifugation (10 min at 9500 RPM, 10 °C). The bacterial pellet was lysed under native conditions, using the CelLytic™ B Plus Kit (Sigma). The cellular debris was removed by centrifugation (15 min at 14,500 RPM, 4 °C) and the cleared lysate was gradually loaded onto a column containing Ni^{2+}-NTA-agarose (Qiagen). The unbound and non-specifically bound fraction was removed by exhaustive washing with saline-phosphate buffer (300 mM NaCl; 50 mM NaH_2PO_4, pH 8.0) supplemented with 20 mM imidazole. The high-affinity bound protein was eluted with saline-phosphate buffer supplemented with 250 mM imidazole. Elution fractions were examined by SDS-PAGE and the r*Eh*CHT1-enriched fractions were pooled and concentrated by ultrafiltration using a Microsep™ UF spin filter (Pall Co.). The protein concentrate was desalted by gel permeation chromatography, using a PD-10 column (Amersham), and eluted with Tris buffer (20 mM Tris–HCl, pH 8.0). The concentration of the r*Eh*CHT1 was obtained by Bradford micro-assay. Enzyme activity was determined by the chitinase assay [26].

2.4. Chitinase activity assay

The standard assay was carried out by measuring the rate of hydrolysis of the fluorogenic substrate 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotrioside [26]. All reactions (100 μL) were started by adding 1 nanomol of substrate to a 50 mM citrate-phosphate buffer (pH 5.0) containing 10 μg of r*Eh*CHT1. After 5 min at 50 °C (otherwise mentioned in the text), the reactions were stopped with 100 μL of 1 M Gly-NaOH (pH 10.3). The fluorescence was immediately registered using the *Fluoroskan Ascent® FL* microplate reader (Thermo Scientific), with excitation and emission wavelengths of 355 and 460 nm, respectively. The chitinase activity was measured as the increment of fluorescence over 5 min and was expressed as FU min^{-1} μg^{-1}.

Table 1
Strains and plasmids used in this study.

Strains or plasmids	Relevant genotype or features	Source or reference
Strains		
Top10F′	*F′[lacI^q Tn10(Tet^R)] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str^R) endA1 λ^−*	Invitrogen
BL21(DE3)	*F^− ompT gal dcm lon hsdS_B(r_B^− m_B^−) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*	Novagen
BW25113	*F^− Δ(Arad-araB)567 ΔlacZ4787(::rrnB-3) λ^− rph-1 Δ(rhaD-rhaB)568 hsdR514*	CGSC[a]
JW3832-2	BW25113 Δ*dsbA723::kan*	CGSC[a]
JW2861-1	BW25113 Δ*dsbC744::kan*	CGSC[a]
MRB10	BW25113 Δ*dsbA723::frt* (from JW3832-2)	This study
MRB20	BW25113 Δ*dsbC744::frt* (from JW2861-1)	This study
Plasmids		
pET22b(+)	T7 RNA pol regulation, pelB leader (periplasmic expression), ColE1 origin, Amp^R	Novagen
pET22/*Eh*CHITv32	pET22b-based, periplasmic *Eh*CHT1	This study
pCP20	pR/cI857-regulated Flp recombinase, *rep*A101ts origin, Cm^R, Amp^R	CGSC[a]
pBAD33	Arabinose regulation, p15A origin, Cm^R	ATCC[b] [25]
pBAD-PelB-*Eh*CHIT84	pBAD33-based, periplasmic *Eh*CHT1	This study
pBluescript SK-	Lactose regulation, ColE1 origin, Amp^R	Stratagene
pBPelB-*Eh*PDI	pBluescript-based, periplasmic *Eh*PDI (wild type)	[51]
pBRM05	pBluescript-based, periplasmic $EhPDI_{SS\text{-}CC}$ (C44S; C47S)	[19]
pBRM06	pBluescript-based, periplasmic $EhPDI_{CC\text{-}SS}$ (C160S; C163S)	[19]
pBRM15	pBluescript-based, periplasmic $EhPDI_{SS\text{-}SS}$ (C44S; C47S; C160S; C163S)	[19]

[a] Coli Genetic Stock Center.
[b] American Type Culture Collection.

2.5. Effects of pH and temperature on recombinant EhCHT1

The optimum pH for r*Eh*CHT1 activity was determined by repeating the assay at different pH values (using a solution of citrate–phosphate–tris–borate as an universal buffer). The stability of r*Eh*CHT1 as a function of pH was examined by assaying the residual activity after an overnight pre-treatment, at room temperature, in universal buffer at different pH values.

The optimum temperature for r*Eh*CHT1 activity was determined by repeating the assay at different temperature values, using the gradient Multigene™ thermal cycler (Labnet International, Inc.). The activation energy for catalysis, E_a (kJ mol^{-1}), was obtained using the linear expression of the Arrhenius equation: $ln(k) = ln(A_0) - E_a\,(1/RT)$, where A_0 represents a pre-exponential factor, R the gas constant (8.314 J°K^{-1} mol^{-1}), and T the absolute temperature (°K). The stability of r*Eh*CHT1 as a function of temperature was studied by assaying the residual activity after pre-incubation for 30 min at different temperatures. As the enzyme inactivation followed a first order reaction, the obtained data were used to estimate the values for thermal inactivation rate constant (k) and half-life ($t_{1/2}$). The k values were calculated using the linear expression of the first order equation: $ln(A_t) = ln(A_0) - k\,t$, where A_0 and A_t represents the relative activity values of fully active (untreated) and thermally inactivated ($t = 30$ min) enzyme, while the $t_{1/2}$ values were obtained using the following equation: $t_{1/2} = ln(2)/k$. The activation energy for denaturation, $E_a{}^*$ (kJ mol^{-1}), was estimated using the Arrhenius equation as described above.

2.6. Effect of guanidine hydrochloride concentration on recombinant EhCHT1

The effect of a chemical denaturation on r*Eh*CHT1 activity was analyzed by repeating the assay in the presence of different concentrations of guanidine hydrochloride. Stability of r*Eh*CHT1 as a function of chemical denaturation was determined by evaluating the residual activity after an overnight pre-treatment, at room temperature, in the presence of different concentrations of guanidine hydrochloride. The effect of chemically-induced denaturation on the stability was determined considering a two-state model (active ↔ inactive). The ΔG at equilibrium was calculated using the free energy equation: $\Delta G = -RT\,ln(f_U/f_N)$, where f_N and f_U represent the fraction of active (native) and inactive (unfolded) enzyme at equilibrium; *e.g.*, $f_N = A$ and $f_U = 1 - A$ (where A corresponds to the relative activity). While the relationship of the free energy with the concentration of the chemical denaturant, [D], was estimated using the following equation: $\Delta G = \Delta G^w - m\,[D]$, where ΔG^w represents the free energy of denaturation in water (kJ mol^{-1}), and m the denaturant dependence of the free energy of denaturation (kJ mol^{-1} M^{-1}).

2.7. Homology-based modeling of EhCHT1

The three-dimensional model for *Eh*CHT1 was predicted by homology using a resolved crystal structure [27]. Template searching was performed with the Blast engine from the NCBI server (ncbi.nlm.nih.gov) using the RSCB Protein Data Bank (PDB) as the database. The crystal structure from human chitinase (PDB: 1HKK) was selected as template [28]. A pairwise sequence alignment was generated using the ClustalX software [29]. The three-dimensional structure was predicted using the Modeller software [30]. Model accuracy was assessed using the ModEval engine from the ModBase server (modbase.compbio.ucsf.edu). Inferred model was visualized with UCSF Chimera software [31].

2.8. In vivo oxidative folding of EhCHT1

The dependence of *Eh*CHT1 on oxidative folding was studied by functional expression in the periplasmic compartment of *E. coli* mutants with defective oxidase or isomerase activities. Fresh subcultures (1:100) of the wild type, Δ*dsb*A, and Δ*dsb*C cells harboring the plasmid pBAD-PelB-*Eh*CHIT84 were grown in medium supplemented with chloramphenicol. Wild type cells harboring the plasmid pBAD33 were used as a control. After 2 h, the protein expression was induced with 0.2% arabinose and cells were grown for an additional 4 h. The bacterial cells (from a 1-mL culture) were harvested by centrifugation (2 min at 10,000 RPM). Cell extracts were obtained using the CelLytic™ B reagent (Sigma) as recommended by the manufacturer. Protein concentration was determined by BCA micro-assay. Enzyme activity was determined by performing the chitinase activity assay, using 10 μg of cell extract as enzyme source.

The assistance of *Eh*PDI to the oxidative folding of *Eh*CHT1 was analyzed by functional co-expression in the periplasmic compartment of the *E. coli* mutant with defective disulfide oxidase activity. Fresh subcultures (1:100) of the Δ*dsb*A cells coharboring the plasmid pBAD-PelB-*Eh*CHIT84 plus one of the following coexpression vectors: pBRM05, pBRM06, or pBRM15 [19], were grown in medium supplemented with ampicillin and chloramphenicol. Δ*dsb*A cells coharboring the plasmids pBAD-PelB-*Eh*CHIT84 and pBluescript SK- were used as a control. After 2 h, the protein coexpression was induced with 0.2% arabinose plus 1 mM IPTG, and cells were grown for an additional 4 h. Bacterial pellets and cell extracts as well as protein concentration and enzyme activity were as described above.

2.9. Data analysis

Unless otherwise mentioned, the activity data were obtained from three independent experiments and represented as mean ± standard error. Unpaired t-test or one-way analysis of variance (ANOVA) was used for routine comparison of data sets. When the p value was less than 0.05, the ANOVA was followed by a Tukey multiple comparison test. All statistical analysis was performed using Prism® v. 5 (GraphPad Software, San Diego, CA). The data from dose–response curves were fitted using a nonlinear least-squares regression method.

3. Results and discussion

3.1. Purification of recombinant EhCHT1 as an active enzyme

During infection, *E. histolytica* goes through changing environmental conditions [6,32,33], therefore, is likely to assume that some amebic proteins, as *Eh*CHT1, were selected to withstand such conditions. To test this notion, we used the recombinant enzyme to study the effects of pH, temperature, and guanidine hydrochloride on the activity and stability. Initially, the protein was properly overexpressed in *E. coli* BL21(DE3) cells harboring the plasmid pET22/*Eh*CHITv32. The r*Eh*CHT1 was isolated and purified from bacterial lysates by immobilized metal affinity chromatography, followed by ultrafiltration and gel permeation. Protein purification was verified by SDS–PAGE and confirmed by immunoblotting. As expected, a single band with a molecular mass of 58 kDa, with purity >95%, was observed. Both protein yield and chitinase activity were determined over the purification process (Table 2).

Table 2
Purification of r*Eh*CHT1 produced in *E. coli*.

Enzyme	Total protein (mg)	Total activity (FU min^{-1})	Specific activity (FU min^{-1} mg^{-1})	Fold purification
Crude	255.84	13,361 ± 593	52 ± 2	1
Pure	5.48	8632 ± 123	1574 ± 22	30.27

Activity reactions (100 μL) were started by adding 1 nmol of 4MUF-triacetylchitotrioside to a citrate-phosphate buffer (50 mM final; pH 5.0) containing 10 μg of protein. After 5 min at room temperature, reactions were stopped with one volume of 1 M Gly-NaOH (pH 10.3). Results are presented as mean ± standard error (n = 2).

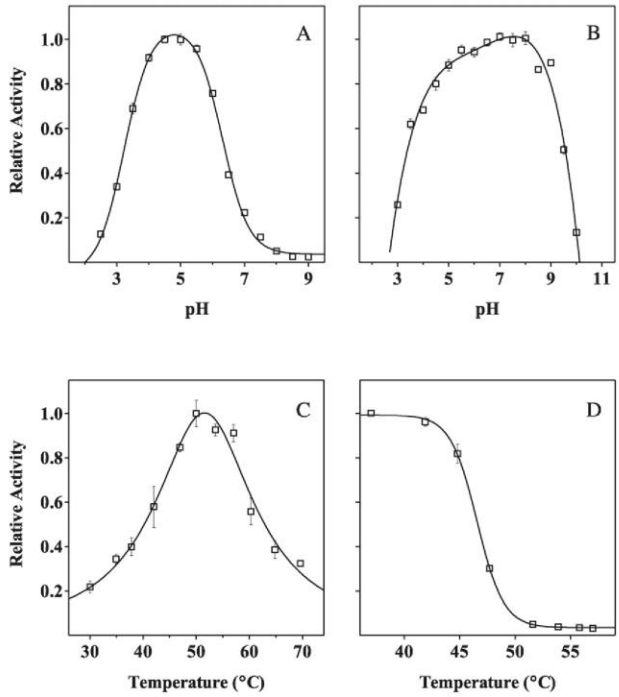

Fig. 1. Activity and stability of recombinant *Eh*CHT1 as function of pH and temperature. Effects of pH on activity (A) and stability (B). Optimum pH for r*Eh*CHT1 activity was determined by repeating the chitinase assay at different pH values. Stability of r*Eh*CHT1 as a function of pH was examined by assaying the residual chitinase activity after an overnight pre-treatment at different pH values. Effects of temperature on the activity (C) and stability (D). Optimum temperature for r*Eh*CHT1 activity was determined by repeating the chitinase assay at different temperature values. Stability of r*Eh*CHT1 as a function of temperature was studied by assaying the residual chitinase activity after pre-incubation for 30 min at different temperatures. Each point represents the mean of three independent experiments.

3.2. The rEhCHT1 is stable over a wide range of pH and temperature

The optimum pH was obtained by repeating the chitinase assay in a solution buffer with different pH values. The r*Eh*CHT1 exhibited maximum activity within pH 4.5 and 5.0 (Fig. 1A), more acidic than those from *E. invadens* chitinases (7.0 to 7.2) [11]. Also, more than 50% of the activity was observed between 3.2 and 6.3, suggesting that the ionization equilibrium reached in the active site is favorable for catalysis [34]. The effect of pH on protein stability was determined by assaying the residual activity after an overnight pH-induced inactivation. The r*Eh*CHT1 remained stable over a wide range of pH, since it showed more than 90% of activity within 5.0 and 8.5 (Fig. 1B). However, a complete loss of activity was noted in extreme values, indicating that deviation from physiological conditions can induce changes in the protonation–deprotonation state of neighboring residues that destabilize the protein structure [35]. Moreover, as the whole process of excystation occurs *in vivo* between the duodenum and cecum of the host [36], the pH conditions prevailing in the lumen of that gastrointestinal segment (fluctuating within 6.0 and 7.4 [37–38]) acts in favor of the *Eh*CHT1 stability.

The optimum temperature was determined by performing the chitinase assay at different temperature values. The r*Eh*CHT1 showed maximum activity at 50 °C (Fig. 1C), higher than those from *E. invadens* counterparts (37 °C for *Ei*CHT1, and 25 °C for both *Ei*CHT2 and *Ei*CHT3) [11]. Any further increase in temperature caused a decrease in activity, suggesting that the active site undergoes thermal unfolding. However, the low activation energy (E_a) estimated for the substrate hydrolysis, 63 ± 2 kJ mol^{-1}, indicates that the active site was correctly folded, promoting the formation of the enzyme–substrate complex and, thus, accelerating the reaction. The effect of temperature on protein stability was estimated by assaying the residual activity after 30 min of thermally-induced inactivation. Although the r*Eh*CHT1 remained fully active after the heat-treatment at 37 °C (Fig. 1D), any further increase in temperature caused a decrease in activity, suggesting inactivation by thermal-denaturation. As first-order kinetics was recognized for the latter, some protein stability parameters were calculated (Table 3). Half-lives for 25 and 37 °C were obtained by extrapolation of an Arrhenius plot. Surprisingly, more than three years were estimated for 25 °C, indicating that

Table 3
Kinetic parameters of r*Eh*CHT1 thermal inactivation.

Temperature (°C)	k (min^{-1})	$t_{1/2}$ (min)
41.9	0.0013	539.2
44.8	0.0067	103.6
47.7	0.0400	17.3
51.6	0.1001	6.9
53.9	0.1084	6.4
55.8	0.1118	6.2
57.0	0.1153	6.0

k, rate constant; $t_{1/2}$, half-life.

high protein stability is prevalent at room temperature. Furthermore, such stability is preserved at physiological temperature (73 h for 37 °C). Moreover, the high activation energy (E_a^*) estimated for thermal-denaturation, 390 ± 55 kJ mol^{-1}, clearly indicates that high energy is needed to start protein denaturation.

Furthermore, compared to other chitinases, *Eh*CHT1 showed some common features exhibited by well characterized bacterial and fungal counterparts, as the optimal values of pH (within 4–9) and temperature (within 40–65 °C), and the thermal stability (up to 40–50 °C) [39–41].

3.3. The rEhCHT1 is sensitive to mild concentrations of guanidine hydrochloride

The effect of guanidine hydrochloride on activity was examined by performing the chitinase assay in the presence of increasing concentrations of denaturant. The r*Eh*CHT1 was significantly inhibited by mild denaturing concentrations of guanidine hydrochloride (IC_{50} of 0.88 M). Furthermore, a complete loss of activity was noted in concentrations greater than 2 M (Fig. 2A), indicating that the active site was fully inhibited under these conditions. The effect of guanidine hydrochloride on protein stability was determined by assaying the residual activity after an overnight of chemically-induced inactivation. The r*Eh*CHT1 was sensitive to mild denaturing conditions, since half-maximal was observed at 0.5 M of guanidine hydrochloride, indicating that irreversible protein denaturation has been occurred (Fig. 2B). Moreover, the high value estimated for the dependence of free energy of stability on denaturant concentration (m), 22.5 ± 1.8 kJ mol^{-1} M^{-1}, clearly suggests a cooperative unfolding process of *Eh*CHT1. Furthermore, it is likely to presume that the presence of the chemical denaturant perturbed both the structure of the protein and the structure of the solvent around the protein [42].

3.4. In silico modeling of EhCHT1 predicts a chitinase-like folding pattern and a putative disulfide bond

So far, our structure–function approach supports the notion that *Eh*CHT1 depends on the correct folding to adopt its native conformation. To extend our knowledge on this issue, a computational approach was performed to predict a three-dimensional model and gain insights into the specific structural features of the protein [43]. After comparative analysis, homology-based modeling was conducted to predict the tertiary structure of *Eh*CHT1 (Fig. 3). The best model showed that the catalytic domain exhibits the typical folding pattern adopted by those from active orthologs, an eight-stranded alpha/beta barrel [44], indicating that *Eh*CHT1 has structural features required for functional performance. Moreover, like many secreted proteins, *Eh*CHT1 contains Cys residues that could form disulfide bonds, a post-translational modification that promotes the correct folding and stabilization of proteins [45]. The mature polypeptide sequence contains 12 Cys residues (UniProt P90546), eight located in the chitin binding domain (CBD), three in the catalytic domain, and one at the C-terminus. Interestingly, it has been established that those residues found in the CBD are forming four disulfide bonds (as it was observed in plant lectins) [46,47]. Furthermore, our 3-D model predicts the formation of an additional disulfide bond between two residues of the catalytic domain (Cys^{167} and Cys^{421}), as their C_β atoms share a distance of 4.2 Å, consistent with the spatial proximity required for disulfide bonding (≤ 45 Å) [48].

3.5. EhCHT1 is dependent on oxidative folding

In light of the above findings, we reasoned that oxidative folding of *Eh*CHT1 is required for proper function within the cell. To address this hypothesis, a simple assay using *E. coli* cells as *in vivo* model was conducted (Fig. 4). Initially, the absence of chitinase activity (as background) was assessed by analyzing the wild type cells harboring the plasmid pBAD33. Also, the periplasmic expression of *Eh*CHT1 was confirmed, as a significant chitinase activity was detected in wild type cells harboring the plasmid pBAD-PelB-*Eh*CHIT84 ($p < 0.01$). Since prevailing redox conditions support the oxidative folding process of *Eh*CHT1, dependence on either the oxidase or the reductase–isomerase activity was tested by functional expression in Dsb mutant cells. In the periplasmic compartment of *E. coli*, oxidative folding is carried out mainly by DsbA (oxidase) and DsbC (reductase–isomerase) proteins [45]. Interestingly, a defective periplasmic expression was observed in Δ*dsb*A cells, as a significant 4-fold reduction of chitinase activity was detected in Δ*dsb*A cells harboring the plasmid pBAD-PelB-*Eh*CHIT84 ($p < 0.01$). In contrast, no significant change was noted in Δ*dsb*C cells (under same experimental conditions). Therefore, it is clear that *Eh*CHT1 depends on the *in vivo* oxidative folding process assisted by DsbA. Moreover, it is reasonable to suppose that the latter task can be assisted by an amebic oxidase with chaperone-like activity, such as *Eh*PDI [19,49–51].

3.6. EhCHT1 is dependent on the oxidase activity of EhPDI

To test the previous notion, we used the defective periplasmic expression of *Eh*CHT1 in Δ*dsb*A cells as *in vivo* phenotype to find out whether it represents a feasible physiological substrate for *Eh*PDI (Fig. 5). First, the periplasmic expression of *Eh*PDI variants in Δ*dsb*A/pBAD-PelB-*Eh*CHIT84 cells was confirmed by immunoblotting. Also, basal chitinase activity

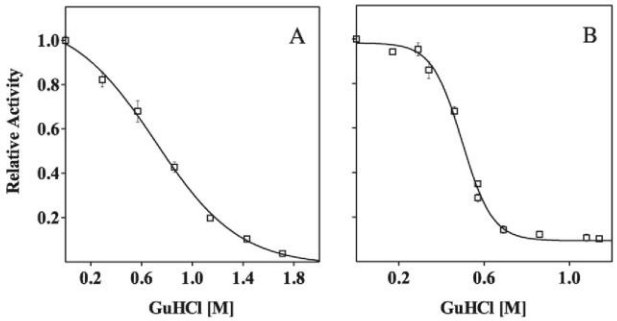

Fig. 2. Activity and stability of recombinant *Eh*CHT1 as function of chemical denaturant concentration. Effects of guanidine hydrochloride (GuHCl) on the activity (A) and stability (B). Activity of r*Eh*CHT1 as a function of the concentration of a chemical denaturant was analyzed by repeating the chitinase assay in the presence of different concentrations of GuHCl. Stability of r*Eh*CHT1 as a function of chemical denaturation was determined by evaluating the residual chitinase activity after an overnight pre-treatment in the presence of different concentrations of GuHCl. Each point represents the mean of three independent experiments.

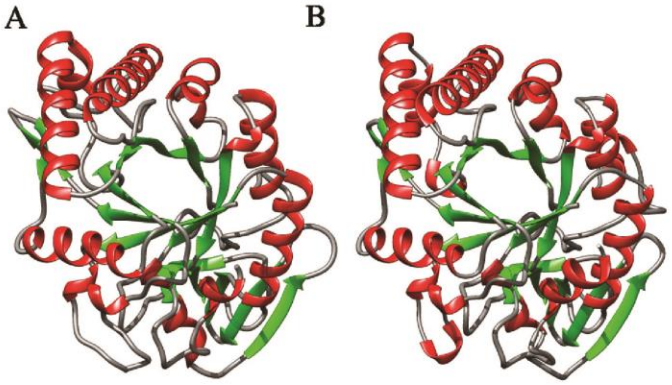

Fig. 3. Predicted structure of the *Eh*CHT1 catalytic domain. Homology-based modeling was carried out by Modeller software using the crystal structure of the human chitinase (PDB 1HKK) as template. Model accuracy was assessed by ModEval engine (ModBase server) and visualized with UCSF Chimera software. Top view of the TIM barrel structure (ribbon representation) of model (A) and template (B). The α-helices are shown in red, while the β-sheets are denoted by green arrows.

was established by analyzing the Δ*dsb*A/pBAD-PelB-*Eh*CHIT84 cells coharboring the plasmid pBluescript SK-. Amazingly, a significant increase of chitinase activity was detected in Δ*dsb*A/pBAD-PelB-*Eh*CHIT84 cells coharboring the plasmid pBPelB-*Eh*PDI ($p < 0.001$), suggesting that *Eh*PDI can assist the oxidative folding process of *Eh*CHT1. The active site of functional thioredoxin-like domains from DsbA and PDI enzymes is characterized by the presence of the motif CXXC, where the Cys residues play an important role in the enzymatic activity [19]. *Eh*PDI contains two thioredoxin-like domains (referred to as N- and C-Trx, respectively; both having the motif CGHC) that are important for the *in vivo* oxidase activity [50]. To validate the latter observation, we conducted a further analysis using *Eh*PDI variants. Interestingly, the Δ*dsb*A/pBAD-PelB-*Eh*CHIT84 cells coharboring the plasmid pRM06 (containing the N-Trx as lone-active domain, $EhPDI_{CC\text{-}SS}$) exhibited full chitinase activity, while those cells coharboring the plasmid pRM05 (containing the C-Trx as lone-active domain, $EhPDI_{SS\text{-}CC}$) showed just 30% of activity, suggesting that both Trx-domains are not functionally equivalent in the oxidative folding process of *Eh*CHT1. Furthermore, a complete loss of chitinase activity was noted in Δ*dsb*A/pBAD-PelB-*Eh*CHIT84 cells coharboring the plasmid pRM15 (containing both Trx-domains as inactive, $EhPDI_{SS\text{-}SS}$), confirming that *in vivo* oxidative folding of *Eh*CHT1 is dependent on the assistance of *Eh*PDI ($p < 0.01$).

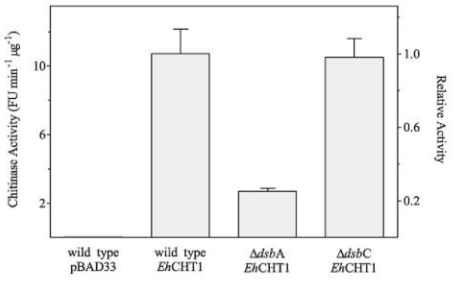

Fig. 4. Amebic chitinase activity when *Eh*CHT1 was expressed in the periplasmic compartment of the wild type (BW25113), Δ*dsb*A (MRB10), or Δ*dsb*C (MRB20) strains. Fresh subcultures of cells harboring pBAD-PelB-*Eh*CHIT84 were grown for 2 h before induction with 0.2% arabinose (wild type cells harboring pBAD33 were used as a control). Protein expression was allowed by cell growing an additional 4 h. Bacterial cells from one milliliter were harvested by centrifugation and total cell extracts were obtained using the CelLytic™ B reagent (Sigma). Protein concentration was determined using BCA micro-assay. Enzyme activity was determined by performing the chitinase assay with 10 μg of total cell extract as enzyme source. The relative activity is shown on the right. Each bar represents the mean of three independent experiments.

4. Conclusions

Recombinant amebic chitinase (r*Eh*CHT1) is functionally active and stable in a wide range of pH and temperature, however it is sensitive to chemical denaturation. Furthermore, *in vivo*, its native conformation is dependent on oxidative folding, a cellular process that may be catalyzed by an oxidoreductase with chaperone-like activity, such as the amebic PDI (*Eh*PDI). Our structure–function approach revealed important features that can be exploited to design specific inhibitors with anti-amebic activity.

*Eh*CHT1 is structurally and functionally similar to chitinases of *E. invadens*, which play a key role in encystation and excystation [10,11]. Hence, it is feasible to assume that *Eh*CHT1 represents a potential target for arresting life cycle of *E. histolytica*, a notion that has been experimentally supported by delaying encystation of *E. invadens* with

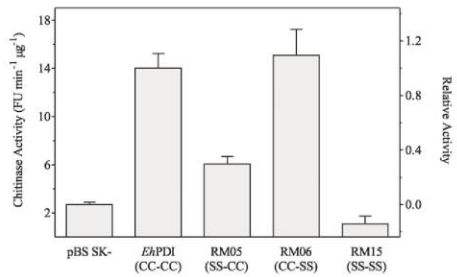

Fig. 5. Amebic chitinase activity when *Eh*CHT1 was co-expressed with *Eh*PDI in the periplasmic compartment of the Δ*dsb*A strain. Fresh subcultures of cells coharboring pBAD-PelB-*Eh*CHIT84 and pBRM05, pBRM06, or pBRM15 were grown for 2 h before induction with 0.2% arabinose and 1 mM IPTG (cells coharboring pBAD-PelB-*Eh*CHIT84 and pBluescript SK- were used as a control). Protein coexpression was allowed by cell growing an additional 4 h. Bacterial pellets and cell extracts as well as protein concentration and enzyme activity were as in Fig. 4. The relative activity is indicated on the right. Each bar denotes the mean of three independent experiments.

the chitinase-specific inhibitor allosamidin [9]. As a potential clinical use, the expected outcome of blocking encystment will be the excretion of trophozoites and immature cyst forms, which will succumb to harsh environmental conditions (due to the lack of a full protective cell wall) [36,52].

Finally, treatment of human amebiasis is challenged by the limited therapeutic options and the potential development of resistance. For instance, metronidazole remains the most effective treatment option and its resistance is an emerging clinical concern [22]. Consequently, there is a need for development of further therapeutic approaches aimed at controlling human parasitic protozoan diseases, such as amebiasis, which comprise the discovery and design of novel therapeutic agents targeting essential proteins [53,54].

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Resumen

In-vivo, las proteínas son sintetizadas por los ribosomas como cadenas lineales. Para ser funcionales, deben plegarse y adquirir su estructura tridimensional (conformación nativa). De manera interesante, algunas proteínas pueden obtener su estructura nativa de manera espontánea. Sin embargo, muchas otras no adoptan esa estructura y adquieren un plegamiento incorrecto, exhibiendo una tendencia a agregarse. Ante esto, el RE contiene una maquinaria molecular que previene la agregación y promueve el plegamiento correcto, controlando la proteostasis celular. Sin embargo, algunas proteínas fallan en su intento de adoptar su conformación nativa, representando una amenaza constante para la función y viabilidad de la célula.

Las infecciones parasitarias son un problema importante de salud pública en regiones tropicales y subtropicales. Diversos estudios enfocados en la biología de protozoarios parasíticos de importancia médica, como *Leishmania*, *Trypanosoma*, y *Plasmodium*, han revelado diversos procesos relacionados con su estilo de vida particular. Dado que soportan condiciones medioambientales adversas conforme avanzan en su ciclo de vida; contendiendo con al menos cuatro tipos de estrés celular: térmico, nutricional osmótico y oxidativo, estos microorganismos han desarrollado diferentes mecanismos de respuesta, los cuales incluyen la activación de la expresión de chaperonas moleculares que juegan papeles importantes, tanto en el mantenimiento de la homeostasis como en el plegamiento correcto de proteínas.

En el presente capítulo, se revisaron los avances actuales sobre el papel funcional que desempeñan las chaperonas y plegasas de protozoarios parasíticos de importancia médica en los mecanismos de respuesta a condiciones de estrés. De igual manera, se analizaron los estudios que proponen a estas moléculas como dianas para el diseño de nuevos agentes anti-parasitarios.

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Chapter 1

PROTEIN FOLDING AND MOLECULAR CHAPERONES OF PROTOZOA

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ABSTRACT

In vivo, proteins are synthesized as linear chains by ribosomes. To be functional, must fold and get their three-dimensional structure (native conformation). Interestingly, some proteins can get the native conformation spontaneously; however, many others cannot and are likely to get a misfolded conformation. Therefore, to facilitate the folding process, the endoplasmic reticulum contains a molecular machinery that includes a set of chaperones, which prevents protein aggregation and promotes correct folding, thereby controlling cellular proteostasis. Despite this, some proteins fail to adopt a native conformation and are more likely to form aggregates, which represent a constant threat to cell function and viability. For instance, human amyloid diseases are characterized by the accumulation of unfolded proteins which form insoluble aggregates. Parasitic infections are a major public health issue

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in tropical and subtropical regions. Several studies focusing on the biology of medically-important protozoan parasites, such as Leishmania, Trypanosoma, and Plasmodium, have revealed processes related to their particular lifestyle. As they endure harsh environment conditions during its life cycle (e.g., dealing with at least four types of cellular stress: thermal, nutritional, osmotic, and oxidative), protozoan parasites have developed a response mechanism, which includes activation of molecular chaperones, to avoid unfolding of proteins. Consequently, molecular chaperones play a key role in both maintaining protein homeostasis and promoting correct folding of virulence-related proteins. This chapter reviews the current advances on the functional role of molecular chaperones and foldases from medically-important protozoan parasites in their mechanisms of response to stressful conditions, as well as an overview of studies focusing on these key molecules as potential targets for designing novel anti-parasitic drugs.

1. INTRODUCTION

Proteostasis, or protein homeostasis, is mainly referred to the cellular processes involved in regulation of the equilibrium between the synthesis, folding, unfolding, and degradation of proteins. Interestingly, molecular chaperones and foldases (folding enzymes) play key roles in those processes. *In vivo*, protein folding is driven by the concerted action chaperones and foldases, as they assist nascent polypeptides to adopt their correct three-dimensional structure. This task is not easy, since there are a wide range of possible conformations that can be adopted, especially in a conglomerated intracellular environment, affecting the selection of the correct protein folding path. So, to circumvent any detour, chaperones and foldases act cooperatively to remodel and reactivate their polypeptide substrates.

In cells, as all processes occur simultaneously, thigh regulation mechanisms must be active to preserve protein homeostasis. An imbalance in these processes could lead to accumulation of aberrant isoforms (unfolded and misfolded polypeptides), which promote the formation of aggregates, resulting in loss of function, cellular stress and, ultimately, cell death. Chaperones and foldases respond to these stressful conditions, assisting protein folding and, thus, preventing any further outcome. So far, the most studied are two heat shock proteins (Hsp70 and Hsp90), an oxidoreductase (protein disulfide isomerase, PDI), and a lectin (calreticulin, CRT).

Chaperones and foldases have been identified in all organisms studied to date, from prokaryotic to eukaryotic cells, including some human protozoan

parasites (Plasmodium, Leishmania, Trypanosoma, Toxoplasma, Giardia, and Entamoeba). As expected, these organisms also depend on protein folding, assisted by chaperones and foldases, to fulfill their cellular functions. Protozoan parasites advance through different stages to accomplish its life cycle. Interestingly, the stage conversion includes several cellular adaptations intended to withstand adverse environmental conditions, from starvation and nutrient depletion to evasion of the host immune system.

Chaperones and foldases play an important role in the development and stage transition of protozoan parasites, promoting the correct folding of proteins involved in cell development, allowing resistance to unpleasant conditions and, thus, ensuring the success of the infection. Furthermore, for some intracellular parasites, such as Plasmodium, chaperones have been also associated with pathogenesis, suggesting a possible selection and expansion of its functions. Therefore, chaperones and foldases represent potential targets for the development of new or improved anti-parasitic drugs.

For instance, the antibiotic geldanamycin binds to the ATP binding site of the Hsp90 counterpart from several parasites and, as a consequence, ATP hydrolysis is inhibited. Since this activity is essential for assisting correct folding, some vital functions are interrupted, such as cell growth and proliferation. Furthermore, geldanamycin inhibits more efficiently chaperones from parasites than those from human. Albeit it has potential as an anti-parasitic agent, geldanamycin cannot be regarded as suitable for human therapy because it exhibits hepatotoxicity. Nonetheless, this compound has served as core to obtain derivatives with reduced toxicity, but keeping its selectivity.

In addition to Hsp90, other chaperones and foldases, such as Hsp40, Hsp70, CRT, PPI, and PDI, have potential as targets for development of anti-parasitic drugs. However, more studies leading to the discovery of new or improved chemotherapeutics are required; specially, targeting medically-important parasites.

2. Molecular Chaperone Machinery and Protein Folding

Cellular proteostasis, as a quality control mechanism, includes the regulation of protein synthesis, folding, unfolding, and degradation. Interestingly, chaperones and foldases are key players in maintaining this

cellular equilibrium (Saibil 2013), since they participate in protein folding, a main process that is coupled to protein synthesis (Kim 2015).

During protein synthesis, the polypeptide folds into a native conformation (tertiary structure) as it emerges from the ribosome; however, many small proteins fail in their attempt to adopt a correct folding and remain as denatured polypeptide (Kim 2015). Two possible mechanisms have been proposed to describe the achievement of the native structure. The first comprises the formation of an initial core structure, from whom the remaining polypeptide chain adopts its correct folding. While in the second, regions of the polypeptide fluctuate in the formation of a secondary structure; then, an intermediate will be formed by joining altogether, which eventually develop the native structure (Efimov 2014).

In contrast, by using mechanical strength, chemical denaturation, and point mutations, it has been demonstrated that acquisition of the protein native structure involves a complex energy landscape in a composite cellular environment, where proteins are exposed to many disturbances that may strongly influence the selection of the folding path (Guinn 2015). Moreover, it has also been shown that during translation, the rate at which each amino acid is incorporated into the nascent polypeptide affects the probability of a protein to acquire its native conformation; therefore, protein folding during translation is hardly balanced (Caniparoli 2015).

Furthermore, to overcome difficulties in the protein folding pathway and to prevent aggregation, chaperones and foldases cooperate and regulate each other to reshape and reactivate substrate polypeptides. In cells, the existing combinations of chaperones and foldases determine the efficiency and specificity of the molecular folding machinery (Reidy 2014).

2.1. Heat Shock Proteins

The best preserved mechanism to protect cells from misfolded proteins is the refolding mediated by evolutionarily conserved heat shock proteins (Hsp). Hsp are induced in response to several types of stress, such as heat shock, nutrient deprivation, or mechanical damage. Hsp recognize unfolded or misfolded proteins and facilitate their refolding either by a mechanism dependent or independent of ATP (Rosenberg 2015; Brüning 2015).

Small Heat Shock Proteins (sHsp)

The sHsp family of chaperones is characterized by their low molecular weight (12-43 kDa) and by containing a conserved α-crystalline domain. This chaperone family is found in a wide variety of cell types, from prokaryotes to eukaryotes, with a number of members ranging from 1 to 19. Interestingly, sHsp assist protein homeostasis by preventing or reversing aggregation under conditions of proteotoxic stress. This function is carried out through an ATP-independent activity, as holdases, by binding partially unfolded polypeptide to an assembly of sHsp oligomers. Then, when conditions are optimal, polypeptide substrates are released for refolding with the assistance of other ATP-dependent chaperones (Liu 2015; Zhang 2015).

Hsp60 Family

The eukaryotic Hsp60 chaperone, mainly found in the mitochondria, is constitutively expressed under normal conditions and is induced by several types of stress, such as heat shock, oxidative stress, and DNA damage. Together with its cofactor Hsp10, Hsp60 is the main machinery for the correct folding of mitochondrial proteins. Moreover, Hsp60 has been found in other cellular compartments, such as the cytosol and plasma membrane; also, it may be exported to the extracellular space, through vesicle-mediated secretory pathway. In the extracellular environment, Hsp60 interacts with receptors present on immune cells and reach the bloodstream, where it acts as a chaperokine (a chaperone with cytokine activity) at distant places (Asea 2000). Furthermore, Hsp60 plays several important roles in some human diseases, being a reliable biomarker for following patients with temporal lobe epilepsy as well as for evaluating cardiovascular risk (Cheng 2014; Gammazza 2015).

In the bacteria *Escherichia coli*, the chaperonin GroEL (a 57 kDa protein) exists as a 14-meric cylinder consisting of two heptameric rings. Together with its co-chaperonin GroES (a 10 kDa protein), existing as a single 7-meric ring, GroEL favors protein folding through an ATP-dependent process. The molecular complex GroEL/GroES binds to a large variety of polypeptide substrates, ranging from 2 to 100 kDa. The apical domains of the GroEL subunits, forming the ring opening, expose the hydrophobic residues for binding of substrates. Upon ATP binding to GroEL, GroES covers the GroEL ring that holds the substrate, generating a closed compartment large enough to contain polypeptides of up to 60 kDa. In this conformation, the substrate is folded during the hydrolysis of seven molecules of ATP in the GroEL ring. The binding of ATP to the opposite ring causes the dissociation of GroES and the subsequent release of the substrate. If the substrate polypeptide has not yet

been fully folded, it is recaptured for another round of folding inside GroEL (Georgescauld 2014).

The CCT chaperone, also called TRiC (TCP-1 ring complex), has been found in mammalian and yeast cells. To assist protein folding, sixteen CCT monomers assemble a protein complex, consisting of two stacked rings of eight subunits each. This complex forms two inner chambers where substrate polypeptides are trapped and then folded. CCT subunits are highly conserved; however, they diverge in the binding regions, allowing functional adaptation to a wide variety of substrates, including actin, tubulin, STAT3, and some cell cycle regulators. CCT is essential for de novo folding of about 10% of the newly synthesized polypeptides and, also, for refolding of proteins denatured by cellular stress. Interestingly, substrates for CCT are characterized by the presence of domains with a marked tendency to form β-sheets (Rüßmann 2012; Kasembeli 2014). CTT cycles between open and closed conformations, which are regulated by ATP. The closed conformation is favored by the transition state of ATP hydrolysis. The cavity shaped in CTT provides plenty space for 70 kDa substrate polypeptides; however, it has the ability to fold larger substrates. For the latter, CCT only captures the protein domains that requires folding assistance (Rüßmann 2012).

Hsp70 Family

In bacteria, the DnaK chaperone (a prokaryotic homolog of Hsp70) plays a major role in misfolding of proteins, preventing aggregation, assisting refolding, and remodeling large aggregates (Evans 2012; Cho 2015). DnaK has two functional domains: the N-terminal ATPase domain (NBD) and the C-terminal substrate binding domain (SBD). The association or dissociation of the substrate is restricted by the nature of the nucleotide bound to the NBD. When ADP is bound (closed conformation), stable interactions with the substrate are favored, while the affinity is reduced when ATP is bound (open conformation) (Evans 2012; Doyle 2015). DnaK performs a wide variety of activities through association with co-chaperones or other chaperones. For instance, the ATPase cycle is controlled by the co-chaperones, DnaJ and GrpE. DnaJ (a member of the Hsp40 family) stimulates ATPase activity, whereas GrpE, a nucleotide exchange factor (NEF), promotes the release of ADP. Moreover, by association with NBD of DnaK, the ClpB chaperone (a member of the Hsp100 family) is activated, facilitating its ability to solubilize and reactivate aggregated proteins (Doyle 2015).

Other members of the Hsp70 family are the cytosolic Ssa and Ssb chaperones. Activated in response to a wide variety of stressor, the main

function of Ssa proteins is to prevent aggregation of denatured polypeptides, by binding to their hydrophobic regions and preventing the generation of amorphous aggregates (Hasin 2014). Ssa proteins are also involved in protein translation, translocation, and folding. Moreover, cells Ssa-deficient exhibit multiple defects, including cell cycle arrest in G_2/M phase, accumulation of precursor proteins targeted to the endoplasmic reticulum (ER) or mitochondria, abnormal nuclear distribution, and aberrant microtubule formation. As other members of the Hsp70 family, Ssa proteins are associated with other co-chaperones, such as Ydj1 (a DnaJ homolog) and Fes1 (a NEF-like protein) (Shaner 2005).

In contrast, Ssb proteins are associated with both the ribosome and the nascent polypeptides emerging from the ribosomal exit channel. Cells lacking Ssb proteins exhibit enhanced sensitivity to inhibitors of translation, indicative of its role in the process. In addition, the ribosome-associated complex (RAC), a heterodimer comprised of Ssz-1 (Hsp70-like) and Zuo-1 (a J-protein), specifically activates the ATPase function of Ssb proteins (Shaner 2005). Therefore, it is likely that RAC and Ssb play key roles in cellular protection of toxic protein aggregates (Kiktev 2015).

A well-studied member of the Hsp70 family is the immunoglobulin heavy-chain-binding protein (BiP), an ER-resident chaperone that distinguishes and binds to unfolded regions of nascent polypeptides during translation. BiP is the main system for monitoring protein folding and participates in various critical processes, such as protein translocation, disaggregation, degradation (Behnke 2015). Also, BiP performs multiple cycles of binding and release of polypeptide substrates to promote proper folding.

This process is tightly regulated by a cycle of ATP binding and hydrolysis as well as by nucleotide exchange (Griesemer 2014; Behnke 2015). Furthermore, some cofactors regulating BiP activity, named ERdj proteins (similar to the bacterial DnaJ, located at the ER), directly bound to unfolded proteins and target this substrate to BiP. In addition, GRP170 and SiL1 (nucleotide exchange factors) facilitate the substrate release from BiP by stimulating the release of ADP and allowing ATP to rebind to the nucleotide binding domain, promoting a conformational change that favors the open state of BiP and allowing accessibility to the substrate binding domain (Araki 2011; Behnke 2015).

Hsp90 Family

A well-studied Hsp90 family member is the Grp94 chaperone, a 94-kilodalton glucose regulated protein, which is the most abundant glycoprotein in the ER (Zhu 2015). Besides participating in protein folding, Grp94 interacts and associates with other chaperones as well as is involved in storage of calcium and assists in targeting of misfolded proteins to the mechanism of ER-associated degradation (ERAD) (Eletto 2010; Marzec 2012). Interestingly, Grp94 substrates are selected, some of which play critical roles in immunity, growth, signaling, and cell adhesion (e.g., major histocompatibility class I, insulin-like growth factor II, Toll-like receptor I, and a subset of integrins) (Liu 2008; Eletto 2010; Staron 2010). Also, Grp94 exhibits a surface and secreted forms that facilitate antigen presentation and immune response (Luo 2013).

Another Hsp90 family member is the mitochondrial TRAP1 chaperone (tumor necrosis factor receptor-associated protein 1), which protects the cell from apoptosis induced by reactive oxygen species (ROS) and senescence (Im, 2014). Recently, it has been showed that TRAP1 is involved in the association of hexokinase II to the mitochondrial outer membrane and promotes the folding and stability of the succinate dehydrogenase, a subunit of complex II from the oxidative phosphorylation chain (Lisanti 2014).

2.2. Calnexin and Calreticulin

Calnexin (CNX) and calreticulin (CRT) are ER lectin chaperones involved in folding and quality control of glycoproteins. CRT is a type I membrane protein, while CNX is its soluble paralog having 39% of sequence homology. CNX and CRT share similar structural features: both have the N-terminal globular domain containing the lectin binding site. In CNX, the globular domain is formed by a β-sandwich of concave and convex β-sheets (Tannous 2015).

In the ER compartment, glycoprotein folding, transport, and degradation is coupled to the N-glycosylation process. An oligosaccharide is transferred to asparagine residues in acceptor polypeptides. Then, glycosidase enzymes modify the branching pattern, leaving a mono-glycosylated glycan, which mediates binding to the ER lectins (CNX or CRT). The latter interaction allows ER-retention of misfolded substrates and, also, promotes refolding through interactions with foldases, such as peptidyl-prolyl cis-trans isomerase

(PPI) and protein disulfide isomerase (PDI) (Ferreira 2004; Banerjee 2007; Ferris 2013; Tannous 2015). Once the substrate is correctly folded, the glycoprotein is transported to the Golgi apparatus. Otherwise, it can either be retained to get another round of refolding or be directed to ERAD (Ferris 2013; Tannous 2015).

2.3. Peptidyl-Prolyl cis-trans Isomerases

Peptidyl-prolyl cis-trans isomerases (PPI) contribute to the folding and restructuring of substrate polypeptides by catalysis of the slow rotational motion of peptide bonds preceding a proline residue. PPI family include parvulins, cyclophilins, and FK-506 binding proteins (FKBP) (Schiene-Fischer 2014). All proteins have a three-dimensional structure where the configuration of each peptide bond is clearly defined. In correctly folded proteins, about 7% of proline bonds have a cis configuration, while non-proline bonds exhibit just 0.03% (Schmidpeter 2015). Hence, PPI promotes folding of partially folded polypeptides by catalyzing the cis-trans isomerization of proline residues with incorrect configuration (Schiene-Fischer 2014; Schmidpeter 2015).

2.4. Protein Disulfide Isomerases

Protein disulfide isomerases (PDI) are oxidoreductases with chaperone activity belonging to the superfamily of thioredoxin-like proteins. Although its primary function is to catalyze the formation and rearrangement of disulfide bonds of polypeptide substrates, PDI enzymes have shown the ability to function as chaperones, a process called oxidative folding of proteins (Stolf 2011; Biran 2014).

Even though they are ER-resident proteins, these enzymes have been found in the cytoplasmic membrane, where catalyze the reduction of disulfide bonds from surface proteins, enhancing adhesion and cell migration. Furthermore, this membrane-associated activity has also been implicated in several processes that regulate host-pathogen interaction during viral and parasitic infections (Santos 2009; Pan 2014).

3. MEDICALLY-IMPORTANT PROTOZOAN PARASITES

Interestingly, a set of chaperones and foldases has been identified in numerous organisms studied so far, including eukaryotic parasites that cause human diseases. Worldwide, parasitic infections are prevalent and remain as a significantly threat to the public health. Tables 1 and 2 summarize the clinical pathology and infective stages of some medically-important protozoan parasites.

Table 1. Medically-important intracellular protozoan parasites

Pathogen	Clinical pathology	Infective stages
Plasmodium	In endemic areas, patients with severe disease usually exhibit one or more of the following: severe anemia, respiratory distress associated with metabolic acidosis, or cerebral malaria. In some cases, patients can acquire partial immunity, allowing the emergence of asymptomatic infections (WHO – Malaria 2015).	The infective stage is named sporozoite. Within the host cell, sporozoites mature into schizonts, then, by asexual replication (schizogony), they release merozoites. When these infect erythrocytes, the ring stage trophozoite matures into schizonts. Some of the intra-erythrocytic trophozoites mature into sexual forms, called gametocytes. Blood stages are responsible for the clinical manifestations of the disease (CDC – Malaria 2012).
Leishmania	Two clinical manifestations can be associated to this parasite infection. Visceral leishmaniasis (VL), characterized by irregular fever, weight loss, hepatosplenomegaly, and anemia. Cutaneous leishmaniasis (CL), characterized by the occurrence of one or more lesions on the skin (face, trunk, or extremities), that can change size and appearance over time (WHO – Leishmaniasis 2015).	The extracellular infective stage, promastigote, develops and multiplies in the digestive tract of the transmitting insects (vectors). The intracellular replicative stage, amastigote, resides and multiplies within the phagolysosomes of the host mononuclear phagocytes (CDC – Leishmaniasis 2013).
Trypanosoma	The lesions produced by the infection of *T. cruzi* (Chagas disease) depend on both parasite and host. Three clinical phases can be distinguished. Acute phase, asymptomatic in about 70% of those infected, characterized by high parasitaemia and multiple tissue invasion. Indeterminate phase, without detectable parasitaemia, where anatomical and functional abnormalities have been reported, and also sudden death. Chronic phase, characterized by irreversible visceral damage (WHO – Chagas 2015).	Trypomastigotes enter the host through a wound or mucosal membranes. Inside, these can invade nearby cells, where they differentiate into amastigotes. These intracellular forms then multiply and differentiate into trypomastigotes, which are released to the bloodstream and spread the infection to other cells and tissues. Within the vector, these trypomastigotes are transformed into epimastigotes, a mobile and replicative stage (CDC – American Trypanosomiasis 2015).

Table 2. Medically-important protozoan parasites associated with diarrheic diseases

Pathogen	Clinical pathology	Infective stages
Giardia	A multifactorial process is associated to the infection with Giardia, which involve functional and immunological aspects of host and parasite. The clinical manifestation range from asymptomatic to acute or chronic disease. The diarrheic syndrome appears as a common acute manifestation (Halliez 2013).	Two stages are clearly distinguished: the cyst, infective form, resistant to the environment outside the host, and the trophozoite, the invasive form, which colonize the small intestine of the host (Halliez 2013; CDC – Giardia 2010).
Entamoeba	The intestinal infection (acute and chronic) is characterized by abdominal pain, tenesmus, and sometimes acute dysenteric diarrhea. The toxic colitis is generated by the invasion and perforation of the colon, which creates a non-specific toxic situation, causing severe peritonitis. The disseminated infection is characterized by invasion of other organs, forming abscesses, the most frequently affected is the liver (Ralston 2011).	The cyst stage, infective form, is resistant to external environmental conditions and can survive in the gastric juice. The trophozoite stage, invasive form, has the ability to attach to the mucosa and colonize the intestinal wall (Fletcher 2012; CDC – Amebiasis 2010).

Protozoan parasites of the phylum Apicomplexa are the most prevalent in the world (Frölich 2011). Within this, parasites of the genus Plasmodium are the most common are the, which cause malaria and are transmitted to humans through the bites of infected Anopheles mosquitoes. There are four species that cause malaria: *P. falciparum, P. vivax, P. malariae* and *P. ovale*. According to the latest global estimates, in 2013, there were roughly 198 million cases of malaria that led to the death of about 584,000 people (WHO – Malaria 2015).

Leishmaniasis is caused by a protozoan parasite of the genus Leishmania, which includes more than 20 different species, and it is transmitted to humans through the bite of infected female sandflies. The disease occurs in three main forms: visceral (the most severe), cutaneous (the most common), and mucocutaneous. Annually, 1.3 million new cases and between 20,000 and 30,000 deaths are estimated (WHO – Leishmaniasis 2015; Alvar 2012).

Chagas disease, also known as American Trypanosomiasis, is caused by the protozoan parasite *T. cruzi*. It is mostly vector-transmitted to humans by contact with feaces of Triatominae bugs. It has been estimated that between 6 and 8 million people are infected worldwide, most of them in Latin America,

where Chagas disease is endemic. Moreover, a total of 56,000 new cases have been reported annually, causing about 12,000 deaths (WHO – Chagas 2015).

WHO reported in 2014 that diarrheal diseases affected more individuals than any other disease associated with infections (WHO – Statistics 2014). In humans, these diseases are caused by different pathogens, including protozoan parasites. *Giardia lamblia*, causative agent of giardiasis, is the most common enteric protozoa with a cosmopolitan distribution (Fletcher 2012). *Entamoeba histolytica*, causative agent of amebiasis, is also a cosmopolitan protozoa, but more prevalent in developing countries with poor sanitary conditions. According to WHO, amebiasis is the third leading cause of death by parasitic infections (WHO/PAO/UNESCO 1997).

4. Proteostasis and Proteotoxic Stress in Protozoa

Numerous and complex biological processes simultaneously occur in living cells. These processes must be strictly regulated to ensure an optimum balance in their functions. Proteins perform a wide variety of cellular functions, including the replication of genetic material, catalysis and metabolism, cell signaling, cell cycle, and membrane transport, among others. Hence, the life of a cell depends on the efficient function of thousands of proteins, which, in turn, depend on correct folding (Cuanalo-Contreras 2013).

Since proteostasis controls protein concentration, subcellular localization, chaperone-assisted folding, and protein degradation, this mechanism is responsible for maintaining the dynamic balance of the cell proteome (Gidalevitz 2010). Therefore, unbalanced proteostasis can lead to aberrant folding, aggregation and accumulation of proteins, causing stressful conditions that may result in cellular dysfunction (Bar-Lavan 2013). Some cells have a high rate of protein synthesis, which makes them more likely to accumulate misfolded proteins and to potentially develop proteotoxic stress (Kawabata 2012).

In eukaryotic cells, the accumulation of unfolded proteins in the ER induces proteotoxic stress, which then triggers a quality control mechanism, the unfolded protein response (UPR) (Hartley 2010). When UPR is efficient, cells can temporarily adapt to that stress (Papa 2012). However, excessive and prolonged ER stress induces cell death, usually by apoptosis, which is the last resort to dispense dysfunctional cells (Xu 2005). Chaperones and foldases that

enhance folding capacity, and initiating the process to export and degrade misfolded proteins. The most abundant and best characterized ER chaperones and foldases include BiP, GRP94, CRT, and PDI. Interestingly, the increased protein synthesis in highly proliferative or secretory cells requires increased ER capacity and function. Therefore, ER chaperones and foldases play important roles in maintaining proteostasis and contributing to cell growth and survival (Hartl 2009; Luo 2013).

Because of ER connectivity to other subcellular compartments, it is possible to comprehend how some chaperones and foldases may be located outside such compartment. For instance, on the cell surface, where they participate in signaling functions that regulate proliferation and cell death (Peters 2011). Furthermore, different studies have shown the potential to regulate ER proteostasis, including the development of specific therapeutics aimed at producing proteotoxic stress in the ER (Liu 2011).

As many other organisms, protozoa also depend on mechanisms that promote efficient protein folding (Becker 1996). Furthermore, for pathogens such as Plasmodium, Trypanosoma, Leishmania, Toxoplasma, Entamoeba, and Giardia, it is essential that virulence factors exported to the plasma membrane or extracellular environment exhibit proper native structure, which is important to fulfill their functions (Joyce 2013; Sibley 2013). Although more information on protein folding in protozoa is needed, the general knowledge on protein homeostasis can be used to design strategies aimed to identify therapeutic molecules with anti-pathogenic activity; specifically, targeting key components of the protein folding machinery, leading to sustained proteotoxic stress, and thus inducing cell death (Calamini 2011; Calamini 2012).

5. Role of Protein Folding in the Life Cycle of Protozoan Parasites

During the course of its life cycle, protozoan parasites advance through different stages that involve morphological and physiological changes, allowing them to successfully perform several processes, such as infecting and invading the host, and surviving harsh environmental conditions (Florence 2002). This developmental adaptation clearly suggests an evolutionary selection that involves the acquisition of nutrients, release of virulence factors, microbial resistance, and effective immune evasion (Khalaf 2012).

Interestingly, the process of infection and invasion exposes the parasite to adverse environmental conditions, such as abrupt changes in temperature and pH, which may affect the stability of proteins (Acharya 2007). Therefore, the assistance of chaperones and foldases is essential to preserve protein homeostasis during the parasitic lifestyle of protozoa.

5.1. Plasmodium

Plasmodium parasites have a complex life cycle that includes several stages (Table 1). Complete cycle is described in greater detail elsewhere (Enomoto 2012; CDC – Malaria 2012; WHO – Malaria 2015). During transmission from the vector to humans, as well as in development within the human cell, the parasite has to confront unfavorable environmental conditions, such as heat stress, e.g., because the temperature in the vector and the human is different, and due to fever episodes in infected humans (Acharya 2007). Chaperones from The Hsp family allow parasite surviving under those conditions, by binding to denatured polypeptides and assisting proper refolding (Shonhai 2007).

The *P. falciparum* Hsp90 homolog (*Pf*Hsp90) plays two major roles in the parasitic lifestyle: i) folding of essential proteins, e.g., virulence factors, and preventing the formation protein aggregates (along with other members of the Hsp family) (Bonnefoy 1994), and ii) modulating the activity of some transcription factors (Banumathy 2003; Kumar 2003). Use of Hsp90 inhibitors has been key to elucidate its participation during infection and development of *P. falciparum*. When *Pf*Hsp90 is inhibited, a large number of Hsp90-substrate polypeptides, which are necessary for development of the parasite within the human erythrocyte, fail to fold properly and undergo degradation (Banumathy 2003; Kumar 2003). Interestingly, this inhibition arrested the parasite progress at an immature stage. However, this condition had no effect on the release of mature forms, which were able to efficiently infect fresh erythrocytes (Banumathy 2003).

Another Hsp-like protein, *Pf*Hsp70, participates in the parasite development during heat stress. The expression of *Pf*Hsp70 is particularly notable in the intra-erythrocyte stages, where is involved in the folding of secreted proteins, granting the settlement of the parasite within the cell (Shonhai 2007). In addition, *Pf*Hsp70 and *Pf*Hsp90, along with some co-chaperones, constitute a multi-chaperone complex engaged in the folding of several proteins (Acharya 2007; Gitau 2012). Moreover, another complex,

formed by *Pf*Hsp70 and *Pf*Hsp40 (a co-chaperone), can function as a refoldase during protein trafficking between the parasite and the erythrocyte (Birkholtz 2008). It seems that *Pf*Hsp40 is important for maturation of the parasite, as the co-chaperone is particularly abundant in this life cycle phase (Achayra 2007).

A large number of proteins involved in virulence and pathogenesis of Plasmodium parasites have cysteine residues forming disulfide bonds, e.g., the apical membrane antigen 1 (AMA-1) or the EGF-like domain of the merozoite surface protein 1 (MSP-1) (Haque 2012). Both proteins AMA-1 and MSP1 depend on accurate oxidative folding to efficiently perform its function (Hodder 1996; Guevara 1997; Moss 2012). In eukaryotic cells, disulfide bond formation is catalyzed mainly by PDI enzymes. In the genus Plasmodium, nine PDI homologs have been described so far (four paralogs in *P. falciparum*) (Haque 2012). PDI enzymes are highly expressed in asexual stages of this parasite (Mahajan 2006). Moreover, it has been shown that oxidative folding of the erythrocyte binding antigen 175 (EBA-175), a virulence factor and leading malaria vaccine candidate, is assisted by a *Pf*PDI enzyme (Gilberger 2003; Mahajan 2006).

5.2. Leishmania

The life cycle of Leishmania parasites comprises two major stages (Table 1). Complete cycle is described in greater detail elsewhere (CDC – Leishmaniasis 2013; WHO – Leishmaniasis 2015). The parasite has developed unique adaptive mechanisms to ensure survival in the harsh environments faced throughout their life cycle (Cunningham 2002). Moreover, to avoid denaturation of proteins, the parasite contains a set of chaperones and foldases playing an important role to ensure successful infection and invasion processes. Within these, some members of the Hsp family have been identified as key in the cellular response to heat shock, by preventing denaturation and aggregation of proteins during infection (Teixeira 2015).

In *L. brazilensis*, the Hsp70 homolog has been reported as one of the major chaperone involved in prevention of aggregation and folding assistance of proteins (Ramirez 2013). Moreover, its biological significance has been confirmed by gene disruption, in *L. intantum* (Folgeira 2008), and knockdown, in *L. donovani* (Raina 2012), leading to defects on cell cycle, growth, morphology, and virulence of the parasite. Interestingly, it seems that thermal stress is required as a signal for parasite differentiation (Clos 1999). Furthermore, it has been suggested that the transient increase in the expression

of heat shock proteins, which precedes the transformation between stages, is associated with the extensive remodeling that accompanies the morphologic transition (Miller 2000).

The Hsp100 and Hsp90 homologs are two additional chaperones involved in Leishmania differentiation. When the parasite is stressed, high levels of Hsp100 are detected (Krobitsch 1999), functioning as integrity protector. Although not decisive for differentiation, it has been shown that Hsp100 is relevant in maintenance of the intracellular stage, as it acts keeping correctly folded essential proteins and some virulence factors (Krobitsch 1999; Clos 2001). On the contrary, Hsp90 is essential for stage differentiation (Wiesgigl 2001). Recently, two isoforms of the p23 co-chaperone has been identified in *L. brazilensis* (Batista 2015). This co-chaperone binds to the Hsp90-ATP complex. Despite having similar affinities for Hsp90, the two isoforms have dissimilar thermal stability profiles and chaperone activities, suggesting that each might be required in different stages of the parasite life cycle.

On the other hand, *L. major* contains a PDI homolog that plays a key role on its parasitic lifestyle. Mutants lacking *Lm*PDI exhibit a non-virulent phenotype, indicating that it may participate in attachment, internalization, or multiplication (Markikou-Ouni 2012). Moreover, strong suppression of parasite proliferation was observed in cultures treated with bacitracin, a known PDI inhibitor (Khalaf 2012). Furthermore, it has been shown that PDI function is crucial for efficient infection of *L. chagasi* to macrophages (Santos 2009). Also, in *L. donovani*, the CRT homolog is important for the targeting of some virulence factors during their traffic through the parasite secretory pathway. Interestingly, altering the chaperone function of CRT affects secretion of acid phosphatases, resulting in a significant decrease in the survival of L. donovani within human macrophages (Debrabant 2002).

5.3. Trypanosoma

Trypanosoma parasites also have a complex life cycle which includes several stages (Table 1). Complete cycle is described in greater detail elsewhere (Nagajyothi 2012; Michaeli 2015; CDC – American Trypanosomiasis 2015; WHO – Chagas 2015). As expected, during transmission and infection, the parasite must be able to withstand harsh environmental conditions and survive the attacks of the immune system, among others. To understand the role of chaperones in the life cycle of Trypanosoma, thermal or ER stress has been used to study the role of several

protein homologs, including Hsp, CRT, and cyclophilin (Paba 2004; Michaelli 2015).

In *T. cruzi*, the Hsp90 chaperone is not involved in stage differentiation. However, inhibition of *Tc*Hsp90 induces a stress response and causes growth arrest, indicating that protein folding is needed to complete the cell cycle of the parasite (Graefe 2002). Additionally, this parasite has four Hsp70 isoforms that are differentially expressed during its life cycle (Shonhai 2007). Furthermore, it has found that some typical chaperone complexes are formed, such as Hsp70-Hsp40 (Edkins 2004).

A major role of protein oxidative folding in Trypanosoma parasites is illustrated by the variable surface glycoproteins (VSG), which are essential for evading the host immune system and maintaining the low level of chronic infection, since they contain several disulfide bonds that are important for structural integrity (Haque 2012). Specific chaperones and foldases responsible for proper folding of VSG remains to be revealed; however, the implication of several Hsp homologs, some PDI enzymes, and the lectin CRT has been proposed (Wang 2010; Field 2010).

5.4. Giardia and Entamoeba

The life cycle of the enteric parasites Giardia and Entamoeba is quite simple (Table 2). Complete cycles are described in greater detail elsewhere (Ralston 2011; Fletcher 2012; Halliez 2013; CDC – Amebiasis 2010; CDC – Giardia 2010). Interestingly, both parasites require the assistance of chaperones and foldases during the synthesis of essential proteins and virulence factors (Ratner 2008; Ramos 2011; Haque 2012).

Cellular functions of the Hsp90 chaperone are associated with parasite development (Debnath 2014). The *G. lamblia* homolog (*Gl*Hsp90) is encoded by two fragments, separated by a large stretch of sequence, which are spliced together in trans (Nageshan 2011; Roy 2012). Apparently, this features helps the parasite to elude natural inhibitors (Roy 2012). Little is known regarding the cellular role of Hsp90 in *E. histolytica* (*Eh*Hsp90): exhibits a weak ATPase activity and is capable of binding to *Eh*Aha-1 (a co-chaperone). Also, it seems that *E. histolytica* is dependent on *Eh*Hsp90 for growth and survival (Singh 2014).

On the other hand, both Giardia and Entamoeba parasites have PDI enzymes involved in protein folding (Haque 2012). In Giardia, two major proteins of the cell wall are targeted to the secretory pathway and are

covalently associated by disulfide bonding (Hehl 2000). Furthermore, PDI activity is crucial in the ER during the differentiation of Giardia parasites. For instance, when disulfide bond formation is inhibited with dithiothreitol, a membrane-permeable reductant, parasite differentiation is irreversible blocked (Knodler 1999). Also, PDI enzymes play an essential role in protein folding of Entamoeba parasites. In *E. histolytica*, some virulence factors that are essential for its parasitic lifestyle require proper oxidative folding to perform their cellular function (Hecht 2004; Chatterjee 2010). Moreover, using *E. invadens* as a model to study the life cycle of Entamoeba parasites, it has been shown that two cell wall proteins, both containing chitin-binding domains stabilized by disulfide bonding, are essential for stage transition (Samuelson 2011). Hence, it has been proposed that a PDI enzyme might catalyze the disulfide boding in amebic proteins involved on its life cycle (Ramos 2008; Mares 2009; Ramos 2011; Mares 2015).

Other foldases, such as cyclophilin and PPI, have been identified in Giardia and Entamoeba parasites. Although participation of cyclophilin in the proliferation of *E. histolytica* has been reported, the specific cellular role in both parasites remains to be elucidated (Ostoa-Saloma 2000; Buchko 2013).

6. Molecular Chaperones As Anti-protozoan Drug Targets

As described so far, protozoan parasites should adapt to different environments throughout their life cycle. They must survive several life-threatening conditions, including sudden changes in pH and temperature as well as different oxygen tension and low-nutrient situations. Also, they have to deal with oxidative stress and immune response in their host. Hence, to resist and survive stressful conditions and complete their life cycle, protozoa have developed a robust molecular machinery, which includes chaperones and foldases as key components (Neckers 2008). Moreover, the potential of these components as targets for the discovery or development of new anti-parasitic is evident (Wiesgigl 2001; Graefe 2002; Kumar 2003; Kumar 2007; Jones 2008; Li, 2009; Mout 2012; Angel 2013; Meyer 2013; Santos 2014).

Among the parasite chaperones and foldases, Hsp90 chaperone has an exceptional potential as a drug target. In protozoan parasites, several inhibitors targeting functions of the Hsp90 counterpart have been reported (Table 3),

being geldanamycin (GA), an antibiotic produced by *Streptomyces hygroscopicus*, the most studied.

Table 3. Inhibitors of Hsp90 homologs from medically-important protozoan parasites

Organism	Inhibitor	Mode of action
P. falciparum	Geldanamycin	Inhibits the ATPase activity. Blocks some stage transitions during development (Kumar 2003; Angel 2013).
	17-AAG[1]	Exhibit affinity for the ATP binding domain, inhibiting its ATPase activity (Kumar 2007; Mout 2012).
	17-PEG-Alkyn-GA[2]	
	Harmine	Exhibits selectivity for the ATP binding site. Inhibits early stage transitions (Shahinas 2012).
	PU-H71	Exhibits high affinity for the ATP binding site, inhibiting its ATPase activity (Shahinas 2013).
L. donovani	Geldanamycin	Inhibits the ATPase activity. Induces cell cycle arrest. Promotes an apoptotic death (Wiesgigl 2001; Li, 2009).
	Radicol	Exhibits affinity for the ATP binding site. Arrest the cell growth (Wiesgigl 2001).
L. brazilensis	Geldanamycin	Inhibits the ATPase activity. Promotes degradation rather than folding (Silva 2013).
	17-AAG[1]	Inhibits the ATPase activity, Promotes arrest of the cell growth (Santos 2014).
L. amazonensis	17-AAG[1]	Inhibits the ATPase activity. Affects the viability of the parasite in the early phases of treatment (Petersen 2012).
T. brucei	Geldanamycin	Exhibits affinity for the ATP binding site. Reduces the cell growth in specific stages (Jones 2008; Meyer 2013).
	Radicicol	Exhibits affinity for the ATP binding site. Inhibits the ATPase activity (Meyer 2013).
	Novobiocin	Exhibits affinity for the C-terminal domain. Induces conformational changes in the chaperone structure (Allan 2006; Meyer 2013).
	17-AAG[1]	Exhibit affinity for the ATP binding domain. Inhibits the ATPase activity. Promotes arrest of growth of specific stages (Jones 2008; Meyer 2013).
	17-PEG-Alkyn-GA[2]	
	17-AG[3]	
	17-DMAG[4]	
T. cruzi	Geldanamycin	Exhibits affinity for the ATP binding domain. Inhibits the ATPase activity. Inhibits cell growth and prevents cell differentiation (Graefe 2002).
G. lamblia and E. histolytica	SNX-2112 and similar	Compete with ATP for the nucleotide binding site (Debnath 2014).
E. histolytica	Geldanamycin	By molecular modeling, exhibits affinity for the chaperone (Singh 2014).
	17-AAG[1]	Exhibits affinity for the ATP binding site. Inhibits the ATPase activity (Singh 2014).

[1]17-Allylamino-17-demethoxygeldanamycin.

[2]17-N-(3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)-pent-4-ynamide-17-demethoxygeldanamycin.

[3]17-(amino)-17-demethoxygeldanamycin.

[4]17-(dimethylaminoethylamino)-17-demethoxygeldanamycin.

GA binds to the adenosine nucleotide-binding domain of Hsp90, competing with ATP for the binding site; thus, inhibiting the hydrolysis of ATP. Also, GA prevents Hsp90 substrate proteins from being correctly folded, and consequently promoting their degradation. By these actions, GA disrupts critical protozoan cell functions, such as growth and proliferation (Li 2009; Meyer 2013; Debnath 2014; Santos 2014). Although GA inhibits Hsp90 through the same mechanism, their cellular effects may vary in different protozoan parasites (Table 3).

In mammalian cells, the IC_{50} of GA for arresting cell growth is roughly 350 μM (Jones 2008). Whilst in protozoan parasites, the drug affects growth in the nM range. This demonstrates the effectiveness of GA as a therapeutic agent for treatment of parasitic infections (Pallavi 2010; Meyer 2013, Singh 2014; Santos 2014). However, the clinical use of GA was not approved, due to the potential risk of hepatotoxicity. Therefore, efforts have been made to obtain GA derivatives with reduced toxicity, while retaining or improving its capacity as anti-parasitic drugs (Kumar 2007; Jones 2008; Mout 2012; Meyer 2013; Li 2015).

Another Hsp chaperone with potential as a drug target is Hsp70. Contrary to Hsp90, few studies have been reported regarding Hsp70 inhibitors targeting the protozoan counterparts. In *P. falciparum*, the marine alkaloid malonganenone A was identified as modulator of *Pf*Hsp70-1 and *Pf*Hsp70-x activities (Cockburn 2011; Cockburn 2014).

Particularly, the *Pf*Hsp70-x sequence contains unique substitutions, often in regions highly conserved in other orthologs, which make it a potential target for anti-malaria drug design (Hatherley 2014).

The ER-resident CRT lectin may also be regarded as a potential target for development of novel anti-parasitic agents. In *T. cruzi*, the CRT counterpart (*Tc*CRT) plays a key role in evasion of the host immune system (Ferreira 2004; González 2015). Similarly, the *E. histolytica* homolog (*Eh*CRT) helps the parasite in its ability to evade the immune system. Specifically, *Eh*CRT inhibits the classical complement pathway, thus protecting the pathogen from host-induced lysis (Ximénez 2014).

The PDI enzymes, a family of foldases, also have potential as targets for the design of new therapeutic agents (Stolf 2011). In *P. falciparum*, eight PDI enzymes have been identified. One of them, P*f*PDI-8, is highly similar to typical PDI enzymes, structurally and functionally. Moreover, it has been shown that some family members are expressed in all developmental stages while other are stage specific, suggesting that a small subset of enzymes, even just one of them, fulfills the main functions of this class of chaperones in the

life cycle of the parasite (Mahajan 2006). In *T. brucei*, two PDI enzymes have been characterized. Although these are not essential for the growth, they play an important role in the defense against the host immune system, by reducing disulfide bonds or by unfolding internalized antibodies to promote rapid proteolysis (Rubotham 2005). In Leishmania parasites, five PDI enzymes were identified in both *L. major* and L. amazonensis (Stolf 2011). Moreover, it has been reported that typical PDI inhibitors, such as bacitracin and *p*CMBA (*p*-chloromercuribenzoic acid), block the enzymatic activity and the parasite growth in culture, suggesting that PDI foldases play a role in the Leishmania development (Hong 2008). Moreover, a PDI foldase from Leishmania has been considered as an antigen for development of vaccines against parasitic infection (Benhnini 2009). In *E. histolytica*, a PDI family of eleven enzymes has been reported (Ramos 2008). One of them, *Eh*PDI, is actively expressed in the infective stage of the parasite, and exhibits the structural features of a typical P5 polypeptide and the functional activities of a classic PDI oxidoreductase (Ramos 2005; Mares 2009; Ramos 2011; Mares 2015). Furthermore, it has been shown that bacitracin inhibits the oxidoreductase activities of *Eh*PDI, suggesting that blocking the function of this foldase can be used as a target for development of alternative anti-amebic drugs (Ramos 2011).

PPI activity has been recognized in three classes of enzymes: cyclophilins, FK-506 binding proteins (FKBP), and parvulins. Cyclophilins are inhibited by cyclosporins and sanglifehrins, FKBP by FK-506 and its analogs, and parvulins by juglone (Lawen 2014). Protozoan parasite counterparts of cyclophilins and FKBP are essential for their life cycle. The anti-parasitic effect of cyclosporine A (CsA) was reported in *E. histolytica*. A dose-dependent inhibition of cell growth was observed when CsA was supplemented to the culture medium. Although the anti-amebic mechanism is not certain, the drug might be inhibiting the isomerase activity of the amebic PPI homolog (*Eh*Cyp) (Ostoa-Saloma 2000). CsA has also a significant inhibitory effect of CsA on the growth of *L. donovani*. This effect clearly suggests an important role for PPI enzymes in the parasite biology. The suppressive action on the host immunity disregards its use as an anti-parasitic drug. However, future research must be intended to identify novel inhibitors that specifically target the PPI enzyme of the parasite (Yau 2010; Yau 2014). CsA also exhibits anti-parasitic activity in *P. falciparum*. Cyclophilins play an important role in the development of this parasite, promoting the export of virulence factors and acting as chaperone in response to heat shock conditions (Marín-Menéndez 2011). Although the anti-parasitic effect of PPI inhibitors

has not been studied in *G. lamblia*, a FKBP-like enzyme (*Gl*FKBP) was already identified (Buchko 2013), supporting the notion that PPI enzymes can be targets for designing drugs with anti-giardial activity.

CONCLUSION

Proteostasis is essential for the proper functioning of any cell, including protozoan parasites. These pathogens have a set of molecular chaperones and foldases that are involved in both the maintenance of the intracellular protein balance and the resistance to adverse environmental conditions (inside and outside their hosts). Also, some of them have been associated with pathogenesis, as they favor the correct folding of important virulence factors, a process that is critical for infection. Although the first studies were conducted more than ten years ago, the search for specific inhibitors directed to block the function of molecular chaperones or foldases from protozoan parasites remains as productive area. Furthermore, the ultimate goal of drug discovery is the rational design of parasite-specific chemotherapeutics. Finally, it is important to highlight that targeting any molecular chaperone or foldase could lead to a success in the drug therapy, since those molecules are located in the center of several protein networks, with diverse functions, which allow the development, establishment, and surviving of the pathogen in adverse environmental conditions.

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