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ESTUDOS TEÓRICOS E DE MODELAGEM MOLECULAR *IN SILICO* APLICADOS À INTERAÇÃO ENTRE A ENZIMA DELTA-AMINOLEVULINATO DESIDRATASE E DISSELENETOS DE DIARILA

TESE DE DOUTORADO

Rogério de Aquino Saraiva

Santa Maria, RS, Brasil

2013

ESTUDOS TEÓRICOS E DE MODELAGEM MOLECULAR *IN SILICO* APLICADOS À INTERAÇÃO ENTRE A ENZIMA DELTA-AMINOLEVULINATO DESIDRATASE E DISSELENETOS DE DIARILA

Rogério de Aquino Saraiva

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Doutor em Bioquímica Toxicológica.

Orientador: Prof. Dr. João Batista Teixeira da Rocha

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elaborada por
Rogério de Aquino Saraiva

como requisito parcial para obtenção de grau de
Doutor em Ciências Biológicas: Bioquímica Toxicológica

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_____
Carmem Juracy Silveira Gottfried, Drª. (UFRGS)

_____
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_____
Paulo César Piquini, Dr. (UFSM)

_____
Simone Pinton, Drª. (UFSM)

Santa Maria, 06 de maio de 2013

Dedico este trabalho aos meus pais Geraldo e Graça, às minhas irmãs Rayane e Regiane, à minha futura sobrinha e à minha namorada Helenicy, que me proporcionaram todo o incentivo, compreensão e apoio para seguir adiante e conquistar meus objetivos, mesmo distante de casa.

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“No one could be a good observer unless he was an active theorizer.”

Charles Darwin

“Rien dans la vie n’est à craindre. Ce n’est qu’à être compris!”

Marie Curie

“If I have seen further it is by standing on the shoulders of Giants.”

Isaac Newton

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

ESTUDOS TEÓRICOS E DE MODELAGEM MOLECULAR *IN SILICO* APLICADOS À INTERAÇÃO ENTRE A ENZIMA DELTA-AMINOLEVULINATO DESIDRATASE E DISSELENETOS DE DIARILA

AUTOR: ROGÉRIO DE AQUINO SARAIVA
ORIENTADOR: JOÃO BATISTA TEIXEIRA DA ROCHA, DR.
Data e Local da Defesa: Santa Maria, 06 de maio de 2013.

A enzima δ-aminolevulinato desidratase (δ-ALA-D) é uma metaloproteína essencial em vários processos biológicos, uma vez que é responsável por catalisar a formação de porfobilinogênio (PBG), um precursor dos tetrapirrólicos (heme, clorofila). Esta enzima é sensível a metais pesados e outros pró-oxidantes e, dessa forma, tem sido classicamente usada como um marcador na intoxicação por chumbo. Estudos *in vitro* e *in vivo* têm demonstrado que o organocalcogênio disseleneto de difenila [$(PhSe)_2$] pode ser um fármaco promissor por demonstrar várias atividades biológicas, incluindo antioxidante, neuroprotetora, anti-inflamatória, anti-aterosclerótica e outras. Por outro lado, o $(PhSe)_2$ e análogos também são tóxicos por inibir a atividade de enzimas sulfidrílicas, incluindo a δ-ALA-D. Baseados em dados experimentais, tem-se especulado que a inibição da δ-ALA-D de mamíferos pode ocorrer via oxidação de dois tióis vizinhos localizados no centro ativo da enzima. No entanto, não se tinha conhecimento de nenhum estudo baseado em modelagem molecular com o intuito de explicar esta interação de forma mais detalhada. Diante disso, objetivamos compreender essas interações a partir da modelagem molecular *in silico*, que consiste em métodos teóricos aplicados para representar ou mimetizar o comportamento e interação de ligantes e enzimas a partir de informações sobre os requisitos estruturais e termodinâmicos essenciais. Os estudos de *docking* molecular indicaram um papel importante das interações π-π envolvendo Phe208 e cátion-π envolvendo Lys199 e Arg209 e anéis aromáticos do (PhSe)2 e análogos bis 4-(clorofenil) disseleneto, bis 4-(metoxifenil) disseleneto e bis 3-[trifluorometil(fenil)] disseleneto. Estas interações permitem uma aproximação entre átomos de Se do composto e –SH da Cys124 (3.3 – 3.5 Å). Os análogos também interagem de forma semelhante com o sítio ativo da δ-ALA-D. De acordo com o método MFCC (Fracionamento Molecular com Capas Conjugadas), foi possível observar interações envolvendo o $(PhSe)_2$ e resíduos posicionados até uma distância de 8,5 Å do centroide do ligante. Phe79, Cys122, Cys124, Pro125, Asp120, Lys199, Lys252 e Cys132 demonstraram as maiores energia de interação (atrativa) com o $(PhSe)_2$. O modelo molecular representado está em conformidade com ensaios *in vitro* e fornece informações importantes que reforçam o mecanismo de inibição especulado. Os grupos fenil do $(PhSe)_2$ são fortemente atraídos por resíduos aromáticos e carregados positivamente presentes no sítio ativo da δ-ALA-D. Dessa forma, permite-se a aproximação da porção eletrófila Se–Se ao grupos nucleófilos $–S^-$ dos resíduos Cys122, Cys124 e Cys132, facilitando a liberação de Zn(II), a oxidação dos tiolatos e a formação de duas moléculas de fenilselenol (PhSeH), levando a consequente inibição da atividade da enzima.

Palavras-chave: toxicologia molecular, organocalcogênios, porfobilinogênio sintase, oxidação de tióis, tetrapirrólicos, *docking* molecular, bioquímica quântica.

ABSTRACT

Thesis of Doctor's Degree
Graduate Course in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria

IN SILICO THEORETICAL AND MOLECULAR MODELING STUDIES APPLIED TO THE BINDING AFFITY OF DIARYL DISELENIDES TO DELTA-AMINOLEVULINIC ACID DEHYDRATASE ENZYME

AUTHOR: ROGÉRIO DE AQUINO SARAIVA
ADVISOR: JOÃO BATISTA TEIXEIRA DA ROCHA, PHD.
Date and Locality of the Defense: Santa Maria, May 6th, 2013.

Delta-aminolevulinic acid dehydratase (δ-ALA-D) is an essential metalloprotein found in several biological processes, since it is able to catalyze the formation of porphobilinogen (PBG), a precursor monopyrol of tetrapyrroles (heme and chlorophyll). This enzyme is sensible to heavy metals and other pro-oxidant agents and, consequently, it has been classically used as a protein marker for lead intoxication. Both in vitro and in vivo studies has shown that the organochalcogen diphenyl diselenide [$(PhSe)_2$] could be a promising drug due to present antioxidant, neuroprotective, anti-inflammatory, anti-atherosclerotic and other activities. Contrariwise, $(PhSe)_2$ could also be toxic because it can inhibit the activity of important sulfhydryl enzymes, including δ-ALA-D. Regarding some experimental data, it has been speculated that mammalian δ-ALA-D inhibition can occur via the oxidation of two vicinal thiols located in it active center site. However, no molecular model had been proposed in order to explain this interaction with details. Thus, we aimed to get a further understanding about the interaction involving δ-ALA-D and diselenides using *in silico* molecular modeling methods, which are consisted in theoretical methods applied in to represent or mimic the behavior and interaction of ligands and enzymes from their structural and thermodynamic information. Docking simulations indicated an important role for π-π interactions involving Phe208 and cation-π interactions involving Lys199 and Arg209 residues with the aromatic ring of $(PhSe)_2$ and analogs bis 4-(clorophenyl) diselenide, bis 4-(methoxyphenyl)diselenide and bis 3-(trifluorometil(phenyl)diselenide. These interactions allowed an approximation between Se atoms and –SH of Cys124 (3.3 – 3.5 Å). The analogs interacted similarly with the active site of δ-ALAD. According to the quantum method MFCC (Molecular Fractionation with Conjugated Caps), interactions involving $(PhSe)_2$ could occur up to 8.5 Å distance from the centroid of active site. Phe208, Phe79, Cys122, Cys124, Pro125, Asp120, Lys199, Lys252 and Cys132 displayed strong attraction energy to $(PhSe)_2$. The representative molecular model is in accordance with *in vitro* assays and gives mechanistic support to previous speculative mechanism of inhibition. Phenyl moieties in $(PhSe)_2$ can be strongly attracted by aromatic and positive charged residues from δ-ALA-D active site. This allows the approximation of the reactive electrophile moiety Se-Se to the nucleophile –S- groups from Cys122, Cys124 and Cys132, facilitating the release of coordinated Zn(II), thiol oxidation and formation of 2 molecules of phenylselenol (PhSeH). In conclusion, the presence of aromatic moieties in $(PhSe)_2$ and its reactive electrophile moiety Se-Se are crucial to δ-ALA-D inhibition, which leads to thiol oxidation and consequent impairment of its activity.

Keywords: molecular toxicology, organochalcogens, porphobilinogen synthase, thiol oxidation, tetrapyrroles, molecular docking, quantum biochemistry.

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MANUSCRITO

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

- **Aminoácidos**

  Ala, A – alanina

  Arg, R – arginina

  Asn, N – asparagina

  Asp, D – ácido aspártico, aspartato

  Cys, C – cisteína

  Gln, Q – glutamina

  Glu, E – ácido glutâmico, glutamato

  Gly, G – glicina

  His, H – histidina

  Ile, I – isoleucina

  Leu, L – leucina

  Lys, K – lisina

  Met, M – metionina

  Phe, F – fenilalanina

  Pro, P – prolina

  Sec, U – selenocisteína

  Ser, S – serina

  Thr, T – treonina

  Trp, W – triptofano

  Tyr, Y – tirosina

  Val, V – valina

- **Elementos químicos, grupos funcionais e fórmulas**

  Ag – prata

  Ag(I) – prata no estado de oxidação 1+

  Aℓ – alumínio

  Aℓ(III) – alumínio no estado de oxidação 3+

  As – arsênio

  Au – ouro

B – boro

Bi – bismuto

Br – bromo

–Bu – butil ou butila

C – carbono

Cd – cádmio

CH…π – interação carbono-hidrogênio – elétrons pi

$–CH_2=$ – grupo metínico

$–CH_3$ – grupo metil

CH_3Hg^{2+} ou $CH_3Hg(II)$ – metilmercúrio

–COOH – grupo carboxila

$–COO^-$ – grupo carboxila desprotonado (carregado negativamente)

Cℓ – cloro

Co – cobalto

Cu – cobre

F – flúor

Fe – ferro

Fe^{2+} – íon ferro no estado de oxidação 2+

Fe^{3+} – íon ferro no estado de oxidação 3+

Fe(II) – ferro no estado de oxidação 2+

Fe(III) – ferro no estado de oxidação 3+

Ga – gálio

H – hidrogênio

Hg – mercúrio

H_2O – água

$HO^{\bullet}$ – radical hidroxil

H_2O_2 – peróxido de hidrogênio

I – iodo

In – índio

Me – metil

MeHg – metilmercúrio

Mg – magnésio

N – nitrogênio

$–NH_2$ – grupo amino

$–NH_3^+$ – grupo amino protonado (carregado positivamente)

NO – óxido nítrico

O – oxigênio

$O_2^{\bullet-}$ – ânion superóxido

$^{\bullet}OH$ – radical hidroxil

–OH – grupo hidroxila

–OMe– grupo metoxil

Pb – chumbo

–Ph – grupo fenil

–R – grupamento variado, radical “R”.

S – enxofre

–S–S – dissulfeto

$–S^-$ – tiolato

Se – selênio

$–Se^-$ – selenolato

–SeH – grupo selenol

–SH – grupo tiol

Sn – estanho

Te – telúrio

Tℓ – tálio

Zn – zinco

Zn(II) ou Zn2+ – íon zinco no estado de oxidação II

- **Outras siglas e abreviaturas**

2D – bidimensional

3D – tridimensional

A – cadeia lateral acética

Å – angström(s) (10^{-10} m)

ALA – ácido 5-aminolevulínico, δ-aminolevulinato ou ácido δ-aminolevulínico

ALAD – gene codificador da δ-aminolevulinato desidratase

ALA-D – δ-aminolevulinato desidratase

ALA-S – δ-aminolevulinato sintase

ALT – alanima aminotransferase

AST – aspartato aminotransferase

ALA – ácido 5-aminolevulínico; ácido δ-aminolevulínico

ANOVA – análise de variância

BIRD – Sítio de ligação, energia de interação e domínio dos resíduos (do inglês *Binding site, Interaction energy and Residue Domain*)

C – citosina

°C – graus célsius

CAT – catalase

CI_{50} – concentração inibitória mediana

DL_{50} – dose letal mediana

DFT – teoria do funcional da densidade (do inglês *density functional theory*)

DOVA – ácido 4,5-dioxovalérico (do inglês *4,5-dioxovaleric acid*)

DTT – ditiotreitol

E – energia

E-Cys124 – cisteína da posição 124 ligada à enzima

FMO – flavina mono-oxigenase

GABA – ácido gama-aminobutírico

GGA – Aproximação do gradiente generalizado (do inglês *Generalized gradient approximation*)

$\hat{H}$– operador hamiltoniano

IC_{50} – concentração inibitória mediana (do inglês median inhibition concentration)

J – joule(s)

K – kelvin

kcal/mol ou $kcal.mol^{-1}$ – quilocaloria(s) por mol

L – litro(s)

LD_{50} – dose letal mediana (do inglês median lethal dose)

LDA – Aproximação de Densidade Local (do inglês *Local Density Approximation*)

m – metro(s)

M – molar (ou mol/L)

$(mCF_3PhSe)_2$ – bis [(3-trifluormetil(fenil)] disseleneto

MFCC – fracionamento molecular com capas conjugadas (do inglês *molecular fractionation with conjugated caps*)

mg – miligrama(s)

mg/kg ou $mg\ kg^{-1}$ – miligrama por quilograma de massa corpórea

mol – mol

mol/L ou mol.L^{-1} – mol por litro

MOPAC – Pacote de Orbital Molecular (do inglês *Molecular Orbital Package*)

nmol – nanomol

P – cadeia lateral propiônica

PBG – porfobilinogênio

PBG-D – porfobilinogênio deaminase

PBGS – porfobilinogênio sintase

$(pCH_3OPhSe)_2$ – bis (4-metoxifenil) disseleneto

$(pC\ell PhSe)_2$ – bis (4-clorofenil) disseleneto

PDB – Banco de Dados de Proteínas (do inglês Protein Data Bank)

PhSeSePh – disseleneto de difenila

$(PhSe)_2$ – disseleneto de difenila

PhSeH – fenilselenol

pK_a – antilogaritmo da constante de equilíbrio

pH – potencial hidrogeniônico, antilogaritmo da concentração de cátion hidrogênio

PM3 – Método de Parametrização 3 (do inglês Parametric Method 3)

QM – mecânica quântica (do inglês quantum mechanics)

QM/MM - mecânica quântica/mecânica molecular (do inglês *quantum mechanics/molecular mechanics*)

QSAR – relação quantitativa entre estrutura química e atividade (do inglês *Quantitative structure-activity relationship*)

R_2 – coeficiente de correlação linear

R-L – receptor-ligante

RMSD – desvio médio da raiz quadrada (do inglês root mean square deviation)

ROS – espécie reativa de oxigênio (do inglês reactive oxygen specie)

s – segundo(s)

sítio A – sítio acético

sítio P – sítio propiônico

SOD – superóxido dismutase

TG – triglicerídeo

UFF – campo de força Universal (do inglês Universal force field)

α – alfa

β – beta

γ – gama

δ – delta

δ-ALA-D / δ-ALAD – delta-aminolevulinato desidratase

ε – épsilon

ε – constante dielétrica

μ – micro

μg – micrograma

μL – microlitro

μm – micrograma

μM – micromolar (= $\mu mol.L^{-1}$)

μmol/kg ou $\mu mol\ kg^{-1}$ – micromol por quilograma

μmol/L ou $\mu mol.L^{-1}$ – micromol por litro

π – pi

π-π – empilhamento de elétrons pi

σ – sigma

Σ – sigma, somatória

Ψ – fi, função de onda

- **Símbolos**

+ mais

– menos

± mais ou menos

× vezes

= igual

> maior que

≥ maior ou igual a

< menor que

≤ menor ou igual a

% por cento

SUMÁRIO

APRESENTAÇÃO

No item INTRODUÇÃO é apresentada uma revisão sucinta da literatura sobre os temas trabalhados nesta tese.

A metodologia realizada e os resultados obtidos que compõem esta tese estão apresentados sob a forma de artigos e manuscrito, os quais se encontram no item RESULTADOS. Nestes artigos constam as seções: Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas.

Os itens DISCUSSÃO E CONCLUSÕES, encontradas no final desta tese, apresentam descrições, interpretações e comentários gerais sobre os artigos científicos incluídos neste trabalho.

As REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem nos itens INTRODUÇÃO, DISCUSSÃO e CONCLUSÕES desta tese.

1 INTRODUÇÃO

1.1 ENZIMA DELTA-AMINOLEVULINATO DESIDRATASE: GENERALIDADES

As porfirinas são uma classe especial de moléculas orgânicas essenciais à execução de vários processos biológicos vitais nos seres vivos (MOCHIZUKI et al., 2010). Estes compostos atuam como coenzimas ou grupos prostéticos de proteínas e, quimicamente, são constituídos por quatro anéis pirróis modificados, geralmente interconectados por ligações metínicas (=CH–) e uma região central contendo um íon metálico bivalente [Fe(II), Mg(II), Co(II), Ni(II)] (JAFFE, 2003; HEINEMANN et al., 2008). Como exemplos mais comuns desta classe de biomoléculas, podemos citar a heme (uma ferroporfirina, figura 1-A), a clorofila (uma magnésio-porfirina, figura 1-B) e a vitamina B12 (uma cobalto-porfirina, figura 1-C).

A heme é um dos grupos prostéticos de proteínas mais importantes, podendo estar envolvida no transporte de oxigênio (hemoglobina e mioglobina), transporte de elétrons (citocromos a, b e c), biotransformação de xenobióticos (citocromo P450) e nos sistemas de proteção contra peróxidos (catalases e peroxidases) (JAFFE et al., 1995). Já a clorofila, de exclusividade dos organismos fotossintetizantes, é responsável pela absorção de luz para obtenção de energia e produção de moléculas orgânicas (MOCHIZUKI et al., 2010).

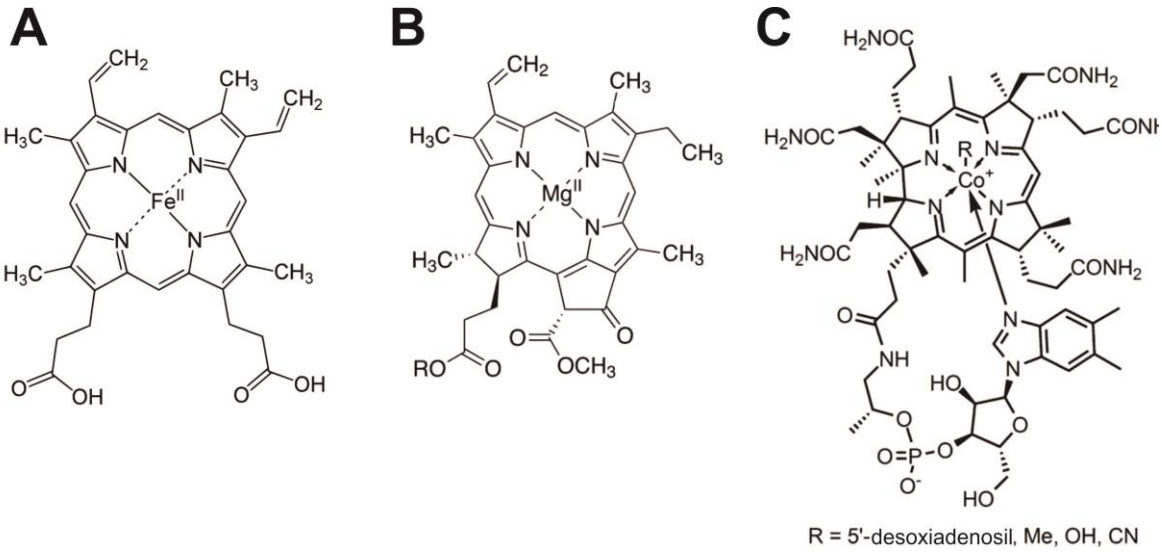

Figura 1 – Estrutura química de porfirinas: heme B (A); clorofila a (B) e vitamina B_{12} (C).

A biossíntese das porfirinas (e de outros compostos tetrapirrólicos) requer, no geral, a atividade coordenada de mais de dez enzimas diferentes (Figura 2), incluindo a enzima delta-aminolevulinato desidratase (δ-ALA-D) ou porfobilinogênio sintase (EC 4.2.1.24). A δ-ALA-D é uma metaloenzima responsável pela síntese de porfobilinogênio (PBG), um monopirrol precursor comum na rota biossintética de todos os compostos tetrapirrólicos, via condensação assimétrica única de duas moléculas de ácido δ-aminolevulínico (δ-ALA) e perda de duas moléculas de água (AJIOKA et al., 2006; NOGUEIRA & ROCHA, 2011). Essa enzima foi isolada pela primeira vez na década de 1950 (DRESEL & FLAK, 1953; GIBSSON et al., 1955).

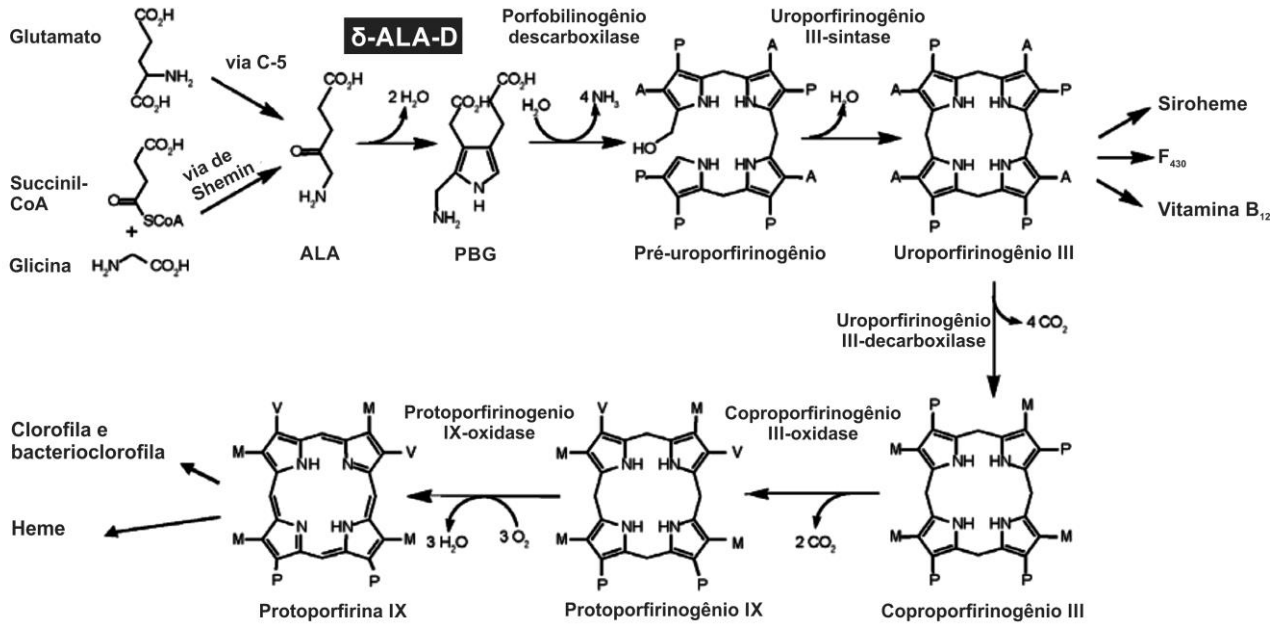

Figura 2 – Rota biossintética dos tetrapirrólicos. Os nomes das enzimas estão apontados sobre as setas. No retângulo preto, é destacada a enzima δ-ALA-D. A: cadeia lateral acética;P: cadeia lateral propiônica; M: grupo metil; V, grupo vinil; ALA: ácido δ-aminolevulínico; PBG: porfobilinogênio. Adaptado de Heinemann et al. (2008).

1.1.1 Estrutura e catálise da enzima δ-ALA-D

Do ponto de vista estrutural, a δ-ALA-D pode existir, em condições de equilíbrio, nas formas octaméricas de alta atividade (com peso molecular entre 280 a 300 kDa), formas hexaméricas de baixa atividade e configurações diméricas alternadas intermediárias (LAWRENCE & JAFFE, 2010), com um sítio ativo por dímero (Figura 3). O sítio ativo apresenta uma configuração tridimensional do tipo TIM-barril (ou α/β barril), onde ocorre o acesso das duas moléculas de δ-ALA.

No geral, o sítio ativo da δ-ALA-D dos seres vivos é bastante conservado. A δ-ALA-D proveniente de animais, leveduras e de algumas bactérias é uma enzima dependente de zinco (CHEH & NEILANDS, 1973; FINELLI et al., 1974), tendo sido demonstrado o envolvimento de resíduos de cisteína na união deste metal (DENT et al., 1990; MITCHELL & JAFFE, 1993; SPENCER & JORDAN, 1994). A enzima proveniente de vegetais, apesar de possuir uma similaridade de 35-50% com a δ-ALA-D de outros organismos, apresenta como importante diferença estrutural a presença de íons magnésio (ao invés de zinco) coordenados a resíduos de ácido aspártico (ao invés de resíduos de cisteína) (SHIBATA & OCHIAI, 1977; TOMAI et al., 1979; BOESE et al., 1991; SCHAUMBURG et al., 1991).

Nos animais e fungos, a δ-ALA-D é encontrada no citosol e a fonte do substrato δ-ALA é mitocondrial (sintetizado a partir de glicina e succinil-CoA, via enzima ALA sintase). Já nos organismos eucariontes e fotossintetizantes (plantas), tanto a δ-ALA-D quanto o substrato δ-ALA são encontrados no estroma de plastídeos (cloroplastos), onde a reação acontece (nesse caso, o substrato δ-ALA é sintetizado a partir de um glutamato carreado por um RNA de transferência RNA_t^{Glu}) (HAMEL et al., 2009; MOCHIZUKI et al., 2010). As demais reações da biossíntese das porfirinas são comuns a todos os organismos.

A ordem e os detalhes dos eventos químicos envolvidos na síntese de PBG pela δ-ALA-D tem sido amplamente avaliados por metodologias variadas, incluindo cinética enzimática, mutagênese direcionada ao sítio, análises das interações do substrato δ-ALA em diferentes estruturas cristalográficas (NANDI & SHEMIN, 1968, JBC; JORDAN ET AL., 1980; JORDAN et al., 1986; JAFFE et al, 2001; FRERE et al., 2002; BREINIG et al., 2003; JAFFE, 2004) e, mais recentemente, por abordagem quântica *in silico* (ERDTMAN et al., 2010). Em todos os estudos, há um consenso de que o grupamento ε-amino de um resíduo lisil do sítio ativo (Lys252 na δ-ALA-D humana) forma uma base de Schiff (–CH=N–) com o C4 da primeira molécula de ALA, a qual origina a cadeia lateral P (propiônica) da molécula de PBG (o grupo amino dessa cadeia lateral se tornará o nitrogênio do anel pirrol). Em seguida, o nitrogênio do grupo amino da segunda molécula de ALA, que vai originar a cadeia lateral A (acética) do PBG, faz interação com o Zn(II) que está coordenado aos tiolatos de três resíduos cisteil (Cys122, Cys124 e Cys132 na δ-ALA-D humana). Ao mesmo tempo, o grupamento ε-amino de outro resíduo lisil do sítio ativo (Lys199 na δ-ALA-D humana) também forma outra base de Schiff com o C4 desta segunda molécula de ALA (correspondente à cadeia lateral A). Conjuntamente, essas interações são essenciais para uma posterior condensação assimétrica das duas moléculas, seguida de liberação de água (Figura 4, ERDTMAN et al., 2010).

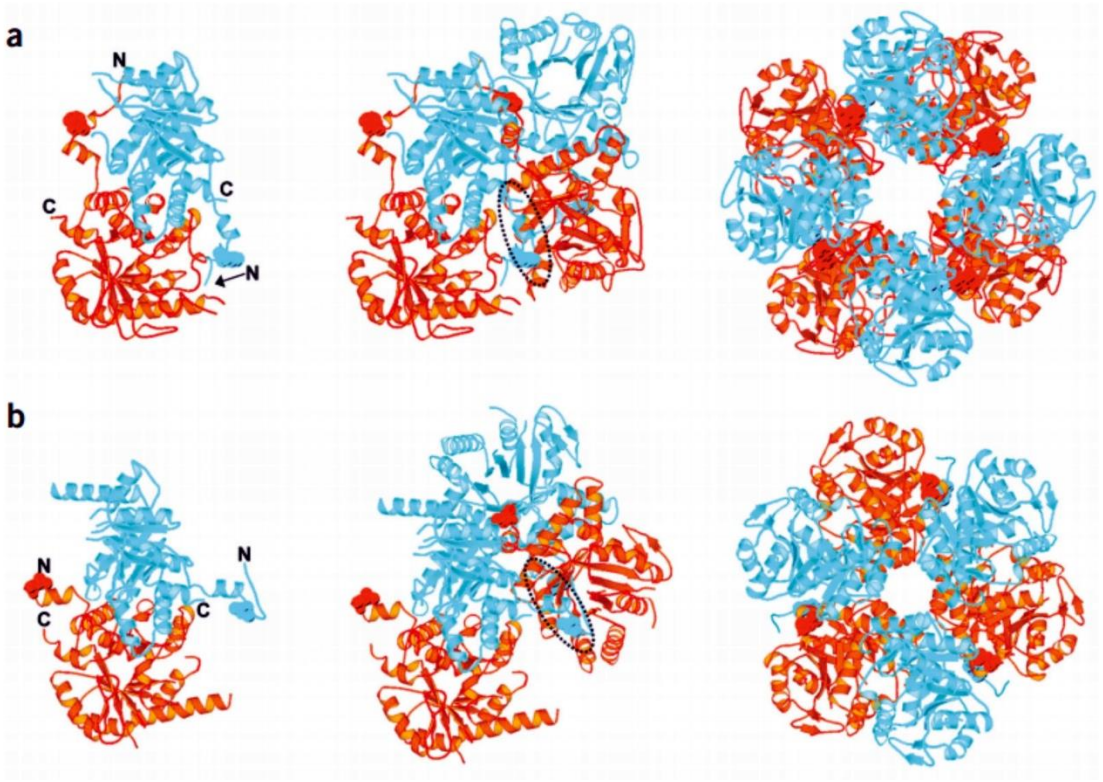

Figura 3 – Estruturas tridimensionais das conformações octamérica (de alta atividade) e hexamérica (de baixa atividade) da δ-ALA-D humana. a- Dímero octamérico (à esquerda) e estrutura octamérica (à direita) (código PDB 1PV8); b- Dímero hexamérico (à esquerda) e estrutura hexamérica (código PDB 1E51). Fonte: Breining et al. (2003).

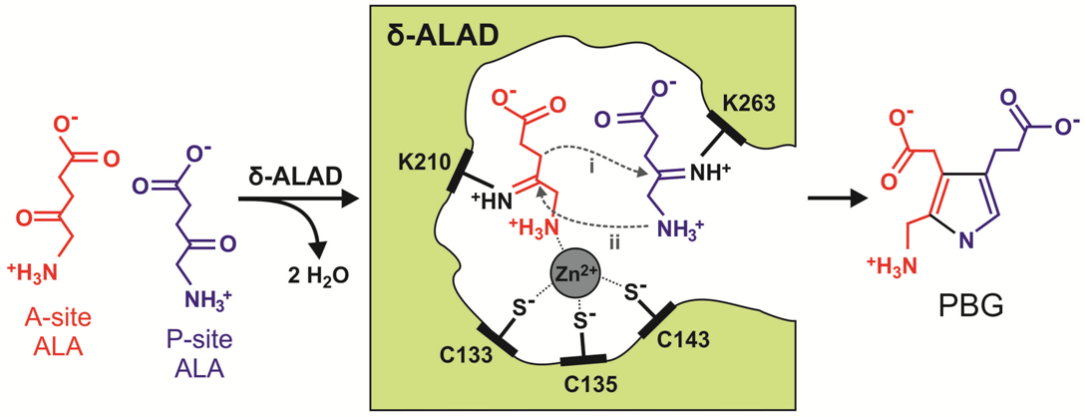

Figura 4 – Esquema do mecanismo catalítico da δ-ALA-D (baseado em cristais da δ-ALA-D de leveduras, códigos PDB 1H7O e 1OHL), demonstrando a condensação assimétrica de duas moléculas de ácido aminolevulínico (ALA) (cadeia lateral A (A-site em vermelho), cadeia lateral P (P-site, em azul) ligadas covalentemente a resíduos de Lys (K210 e K263), formando bases de Schiff. Abaixo, nota-se o zinco (Zn^{2+}) coordenado a três tiolatos (S^-) dos resíduos de Cys (C133, C135 e C143). O esquema sugere um importante papel do Zn no reconhecimento do grupo amino da cadeia lateral A para posterior formação de porfobilinogênio (PBG) e perda de 2 moléculas de H_2O.

Na espécie humana, a enzima δ-ALA-D é codificada por um simples gene localizado no cromossomo 9q34, que apresenta dois alelos codominantes ALAD1 e ALAD2. A natureza desse polimorfismo ocorre devido a uma transversão de uma guanina (G) por citosina (C) no nucleotídeo de posição 177, levando a uma substituição do resíduo Asn59 (na ALA1) para Lys59 (na ALA2). Como a lisina é um resíduo com cadeia lateral carregada positivamente enquanto que a asparagina é um resíduo neutro, a expressão desses alelos resultam em três formas diferentes de isoenzimas, designadas δ-ALA-D1-1, δ-ALA-D1-2 e δ-ALA-D2-2. Sendo assim, os indivíduos heterozigotos ALAD-1/ALAD-2 expressam uma enzima mais eletronegativa quando comparada com indivíduos homozigotos ALAD-1/ALAD-1 e indivíduos homozigotos ALAD-2/ALAD-2 expressam uma enzima mais eletronegativa do que os indivíduos homozigotos ALAD-1/ALAD-1 (KELADA et al., 2001; FUJIHARA et al., 2009). Contudo, essas diferenças de carga entre as isoenzimas não influenciam a atividade na síntese de PBG, uma vez que o sítio ativo não é afetado (JAFFE et al., 2001).

1.1.2 Aspectos toxicológicos

A inibição da δ-ALA-D pode prejudicar a rota biossintética da heme, resultando em consequências patológicas inespecíficas, uma vez que a produção exagerada de espécies reativas de oxigênio pode atuar nos mais diferentes órgãos e compartimentos celulares dos organismos nos quais são gerados (SASSA et al., 1989). Além da insuficiente produção de heme (que pode levar a uma doença conhecida por porfiria), a inibição da δ-ALA-D pode resultar no acúmulo de δ-ALA no sangue, levando à superprodução de espécies reativas de oxigênio (ERO) (BECHARA et al., 1993). Assim, recentemente, tem se utilizado a δ-ALA-D como um potencial bioindicador de estresse oxidativo em condições patológicas (intoxicação por metais, hemodiálise, câncer, diabetes, hipotireoidismo) (GONÇALVES et al., 2005; SOUZA et al., 2007; VALENTINI et al. 2007). Porém estes resultados podem ter variáveis intervenientes relacionadas aos efeitos metabólicos severos causados por tais patologias, o que poderia levar a um possível erro de interpretação (GONÇALVES et al., 2005, 2009; DA SILVA et al., 2007; GROTTO et al., 2010).

A dosagem da atividade da enzima δ-ALA-D tem sido utilizada há muito tempo como um marcador de exposição ao chumbo (Pb) (OSKARSSON, 1989; TAKEBAYASHI et al., 1993; ZHAO et al., 2007). Além disso, indivíduos portadores de alelo ALAD-2 parecem apresentar um maior conteúdo de Pb no organismo e maior risco de intoxicação pelo mesmo,

possivelmente devido a uma maior afinidade da enzima determinada por este alelo (WETMUR, 1994).

Além do chumbo, outros metais (mercúrio, cádmio, berílio) e espécies reativas são capazes de inibir a atividade da enzima δ-ALA-D (SASSA & KAPPAS, 1983; ROCHA et al., 1993; SAKAGUCHI et al., 1997). A atividade da δ-ALA-D de mamíferos é inibida por moléculas quelantes como EDTA e a 1,10-fenantrolina (CHEH & NEILANDS, 1976; SOMMER & BEYERSMANN, 1984). Em mamíferos, esta inibição pode ser revertida pela adição de zinco (BEVAN et al., 1980), demonstrando que a δ-ALA-D requer zinco para estar ativa. O uso de agentes redutores, como ditiotreitol (DTT) e β-mercaptoetanol, também é capaz de reverter a inibição da δ-ALA-D causada por agentes oxidantes de grupamentos sulfridrílicos (JAFFE et al, 2001; JAFFE, 2004).

1.2 COMPOSTOS ORGÂNICOS DE SELÊNIO COMO POTENCIAIS FÁRMACOS

Nos últimos anos, vários grupos de pesquisa têm se empenhado no desenvolvimento de novos fármacos a partir de compostos sintéticos orgânicos contendo selênio (Se), principalmente pela evidência de que essas moléculas podem formar espécies nucleofílicas potentes, que poderiam exercer várias atividades biológicas interessantes (NOGUEIRA & ROCHA, 2011). Apesar de o Se ter sido descoberto em 1817 por Berzelius, sua importância para o sistema biológico só foi descrita cerca de 100 anos depois. De fato, no começo do século XX, o selênio tornou-se notório por sua toxicidade (PAINTER 1941, CAMPBELL et al.,1952, LACASSE & RICHER, 1976), mas sua importância e seu papel biológico ficaram obscuros até 1957. Neste ano, Schwarz e Foltz demonstraram que o selênio podia prevenir uma necrose hepática em roedores alimentados com uma dieta deficiente em vitamina E e selênio (SCHWARZ & FOLTZ, 1957). Todavia, o papel molecular desempenhado pelo Se nos sistemas biológicos só foi esclarecido 15 anos após esta publicação. Em 1973, um grupo europeu e um americano demonstraram que o Se estava associado à enzima glutationa peroxidase, mas a natureza química da interação não era conhecida (FLOHE et al., 1973, ROTRUCK et al. 1973). Cerca de 5 anos depois ficou demonstrado que o Se estava presente nesta enzima na forma química de selenocisteína (FORSTROM et al., 1978) e sugeria que o grupo selenol deste aminoácido teria papel fundamental na degradação de peróxido de hidrogênio.

A selenocisteína é um aminoácido análogo a cisteina e serina, onde os átomos de S ou O são substituídos por Se. Atualmente, cerca de 30 genes para selenoproteínas foram descritos (JOHANSSON et al., 2005) e em todos os casos o selênio está presente na forma de selenocisteína. Todavia, o papel biológico da maioria destas proteínas ainda não é conhecido. Além das glutationas peroxidases, que possuem papel fundamental na degradação de peróxidos, o selênio também faz parte de outras enzimas essenciais na regulação do estado redox da célula, destacando-se a sua participação na tiorredoxina redutase (ARNER & HOLMGREN 2000).

Um dos potenciais candidatos a fármaco contendo Se bastante estudado é o ebselen (2-phenyl-1, 2-benzisoselenazol-3(2H)-one, figura 5-A). Na década de 1980, Sies e colaboradores publicaram uma série de estudos mostrando que o ebselen era um mimético da glutationa peroxidase e podia neutralizar a ação de agentes oxidantes em sistemas biológicos e químicos. Cerca de quinze anos depois da primeira publicação de que o ebselen poderia ser um promissor agente antioxidante para o tratamento de patologias associadas com o estresse oxidativo, o composto foi usado com certo sucesso em 3 estudos clínicos envolvendo isquemia no sistema nervoso. Em estudos com roedores, o ebselen demonstrou ser uma droga efetiva na redução da ototoxicidade e nefrotoxicidade induzida pelo quimioterápico cisplatina (LUO et al. 2003, MUGESH et al. 2001, MAY 2002; LYNCH et al, 2005; NOGUEIRA & ROCHA, 2011).

Outros compostos cujas atividades biológicas tem sido amplamente avaliadas nos últimos anos é o disseleneto de difenila [$(PhSe)_2$] e análogos. Neste contexto, estudos envolvendo vários modelos *in vitro* e *in vivo* têm demonstrado que o $(PhSe)_2$ pode ser considerado um agente terapêutico em potencial devido à evidência de atividade antioxidante (da mesma forma que o ebselen, é também mimético da glutationa peroxidase e pode agir como substrato da tiorredoxina redutase), antiúlcera, neuroprotetora, anti-inflamatória, anti-hiperglicêmica, anti-aterosclerótica e anti-hipercolesterolêmica, dentre outras (DE BEM et al., 2009; DE FREITAS et al., 2009; SAUSEN DE FREITAS et al., 2010; NOGUEIRA & ROCHA, 2011; HORT et al., 2011).

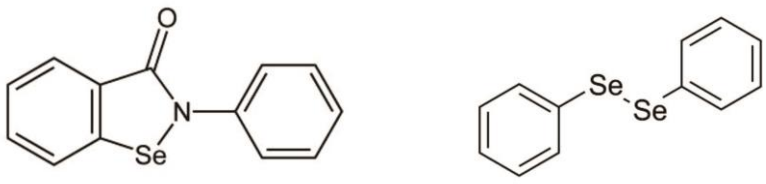

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Figura 5 – Compostos orgânicos de selênio. (1) – ebselen, (2) disseleneto de difenila $(PhSe)_2$

No entanto, de forma oposta, também tem sido demonstrado que o $(PhSe)_2$ e análogos demonstram toxicidade, por apresentar efeitos citotóxicos (POSSER et al., 2011) e inibir a atividade de várias enzimas sulfidrílicas *in vitro*, incluindo a δ-ALA-D (MACIEL et al., 2000; BRÜNING et al., 2009, ARDAIS et al., 2011, KADE & ROCHA, 2010; PUNTEL et al., 2010; STRALIOTTO et al., 2010; LUGOKENSKI et al., 2011).

Tem sido demonstrado que a exposição *in vivo* ao $(PhSe)_2$ inibe a δ-ALA-D de ratos (*Rattus novergicus*), camundongos (*Mus musculus*), peixe jundiá (*Rhamdia quelen*) e da mosca-de-frutas (*Drosophila melanogaster*) por oxidação de seus resíduos sulfidrílicos (BARBOSA et al., 1998; FARINA et al., 2002; SOARES et al., 2004; FACHINETTO et al., 2006; GOLOMBIESKI et al., 2008), mas não a δ-ALA-D do pepino (*Cucumis sativus*), uma vez que na δ-ALA-D de plantas, resíduos de cisteína são substituídos por resíduos de ácido aspártico e o metal Zn(II) é substituído por Mg(II), não havendo a possibilidade de oxidação de tióis catalíticos (FARINA et al., 2002; JAFFE, 2003).

No entanto, é necessária uma continuidade nesses estudos de forma a elucidar de forma mais precisa os mecanismos de toxicidade de organocalcogênios frente à interação com a δ-ALA-D.

1.3 MODELAGEM MOLECULAR *IN SILICO* COMO FERRAMENTA NA AVALIAÇÃO DA ATIVIDADE BIOLÓGICA DE MOLÉCULAS

A evolução de áreas como a fisiologia, a farmacologia, a bioquímica, a química orgânica, a física quântica, ao longo do século XX, puderam, conjuntamente, propiciar uma visão mais íntima da natureza biológica, entendendo-a como resultado de interações entre macro e micromoléculas com funções definidas e que estão em constantes transformações.

Nesse sentido, a modelagem molecular *in silico* (usando ferramentas computacionais) consiste na aplicação de métodos teóricos utilizados para representar ou mimetizar o comportamento e interação de moléculas (RONCAGLIONI et al., 2008). Esta compreensão tem sido considerada uma estratégia promissora na descoberta e planejamento de novos fármacos, uma vez que ela motiva a possibilidade de redução do tempo e dos altos custos envolvidos no desenvolvimento de novos medicamentos (MAGALHÃES et al., 2007).

A modelagem molecular também pode avaliar parâmetros toxicológicos de substâncias frente a alvos importantes (SUN & YOST, 2007) e planejar teoricamente novas

moléculas que satisfaçam as propriedades eletrônicas e estruturais para um perfeito encaixe no sítio receptor (KITCHEN et al., 2004). Em estudos de toxicologia *in silico*, a abordagem é essencial para compreender os mecanismos de interação entre agentes tóxicos e alvos importantes, de forma a permitir um planejamento racional de fármacos com baixa afinidade para esses alvos (MA et al, 2011).

A determinação da estrutura tridimensional de proteínas e outras moléculas por cristalografia de raios-X e RMN, somados ao conhecimento da química molecular e quântica e a aplicação da tecnologia computacional permitiram, conjuntamente, o desenvolvimento de técnicas de modelagem molecular que existem atualmente (MAGALHÃES et al., 2007).

Para que se possam desenvolver estudos de modelagem molecular, existem dois tipos de métodos teóricos aplicados: os baseados em mecânica clássica e os baseados em mecânica quântica (PATRICK, 2007).

Nos métodos baseados em mecânica clássica, a molécula é tratada como uma série de esferas carregadas (os átomos) conectadas por bastões (as ligações), sem considerar a posição dos elétrons. Esses métodos são usados para calcular as diferentes interações e energias (campos de força) resultantes do alongamento das ligações, ângulo de flexão, interações não-ligadas e energias de torção (PATRICK, 2007; MAGALHÃES et al., 2007).

Já a mecânica quântica usa física quântica para calcular as propriedades de uma molécula considerando as interações entre os elétrons e o núcleo de cada átomo, com a aplicação da equação de Schrödinger (equação 1):

$$\hat{H}\Psi=E\Psi \qquad (1)$$

onde, de maneira bastante simplificada, o operador hamiltoniano (**$\hat{H}$**) descreve as energias potenciais e cinéticas entre elétrons e núcleo levando em consideração a posição espacial de cada partícula; o **Ψ** é a função de onda, que é uma função da posição da partícula e do tempo, e o E é a energia total do sistema (ALMEIDA & SANTOS, 2001).

Diferentemente da mecânica molecular, os átomos não são tratados como esferas sólidas. Para sistemas polieletrônicos, os cálculos se tornam mais complexos. Por isso, no intuito de fazer cálculos mais prováveis e executáveis, várias aproximações são feitas. Como exemplos dessas aproximações, podem ser citadas as aproximações de Born-Oppenheimer, onde os núcleos dos átomos são tratados de forma estática, enquanto que os elétrons são tratados de forma dinâmica, e os estudos da Teoria do Funcional da Densidade (DFT), que levam em consideração a densidade eletrônica (TOSTES, 1998).

Do ponto de vista computacional, os cálculos envolvendo métodos clássicos são mais rápidos, porém com uma menor acurácia quando comparado com os cálculos quânticos, já com os cálculos quânticos, ocorre o inverso, maior acurácia e mais lentos computacionalmente. Sendo assim, métodos quânticos são mais adequados para sistemas moleculares menores (ordem de 10 a 100 átomos), enquanto que os métodos clássicos são mais adequados para sistemas maiores (na ordem de 500 a 1000 átomos ou mais) (PATRICK, 2007).

Para o estudo das interações entre ligantes e macromoléculas (receptores proteicos, enzimas, DNA, canais iônicos), o método *in silico* mais utilizado é o de *docking* molecular. O problema de *docking* receptor-ligante consiste basicamente na predição do modo de ligação de uma pequena molécula ligante (inibidor ou substrato) na região de ligação (sítio ativo) de um alvo molecular, além da quantificação da afinidade de ligação entre o receptor e o ligante (KITCHEN et al., 2004; MAGALHÃES et al., 2007).

No geral, os programas de *docking* combinam dois elementos fundamentais: (i) um algoritmo de busca, que são comandos que permitem atuar nos graus de liberdade do sistema ligante-macromolécula para que se conheçam os modos de ligação mais verdadeiros; e (ii) a função *scoring*, que consiste em métodos teóricos (na maioria das vezes clássicos ou com parametrização baseada experimentalmente) usados para predizer a força das interações não-covalentes entre as duas moléculas do sistema (KITCHEN et al., 2004).

1.4 JUSTIFICATIVA DO ESTUDO

Embora estudos tenham demonstrado que o $(PhSe)_2$ e análogos inibem a atividade da δ-ALA-D *in vitro* por oxidação de tióis, até o momento não havia a proposição de nenhum modelo molecular capaz de explicar esta interação mais detalhadamente. Baseados em dados experimentais, Farina e colaboradores (2002) especularam que a inibição da δ-ALA-D de mamíferos poderia ocorrer via oxidação de dois grupos tióis vizinhos localizados no centro ativo da enzima (FARINA et al., 2002). Nesse esquema (ver Figura 2 da página 59), um grupo –SH mais reativo (marcado com um *) deve fazer um ataque nucleofílico em um dos átomos de selênio do disseleneto, formando o primeiro selenofenol (PhSeH). Em seguida, seria esperado do –SH vizinho menos reativo atacar o átomo de enxofre do intermediário E–S*-SePh, formando o segundo selenofenol (PhSeH) e, consequentemente, oxidando os dois tióis vizinhos, fazendo assim com que a enzima seja inibida.

Portanto, nossos estudos têm se concentrado em compreender as interações entre organocalcogênios (disseleneto de difenila e análogos) e enzimas sulfidrílicas por modelagem molecular *in silico* e estudos adicionais *in vitro*, de forma a entender os mecanismos de inibição bem como adquirir informações essenciais que permitam o desenvolvimento de novos fármacos organocalcogênios que mantenham suas propriedades terapêuticas evidenciadas em vários estudos *in vivo* e *in vitro* e, ao mesmo tempo, apresentem baixa afinidade para essas enzimas.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Compreender, de forma mais detalhada, o mecanismo molecular envolvido na inibição da enzima δ-ALA-D pelo $(PhSe)_2$ e análogos, através de estudos teóricos e de modelagem molecular *in silico*.

2.2 OBJETIVOS ESPECÍFICOS

- Analisar o modo de ligação do $(PhSe)_2$ com a enzima δ-ALA-D humana através de *docking* molecular *in silico*;
- Identificar os resíduos do sítio ativo da δ-ALA-D que interagem diretamente com o $(PhSe)_2$, através de cálculos de DFT (método MFCC);
- Explicar, com base nos modelos moleculares obtidos, como ocorre a oxidação dos grupos tióis catalíticos da enzima δ-ALA-D por disselenetos;
- Analisar o grau de homologia entre a δ-ALA-D de diferentes organismos e correlacionar com a atividade inibitória dos disselenetos;
- Correlacionar energia livre de afinidade calculada *in silico* com valores experimentais *in vitro*, bem como a distância entre o átomo de selênio dos disselenetos e o tiolato de resíduos de cisteína do sítio de ligação;
- Contribuir com informações essenciais que permitam o desenvolvimento de novos fármacos organosselenetos com baixa afinidade para a δ-ALA-D.

3 RESULTADOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos (2) e manuscrito (1). Os itens “Materiais e métodos”, “Resultados”, “Discussão” e “Referências Bibliográficas” estão contidos nos próprios artigos e manuscritos. Os **artigos científicos** estão dispostos na forma em que foram publicados nos periódicos *Toxicology Research* e *Journal of Toxicology and Environmental Health-Part A*, respectivamente. O **manuscrito** está disposto na forma em que se submete para publicação no periódico científico *Journal of Theory Chemistry and Computation*.

3.1 Artigo 1: "δ-Aminolevulinato desidratase (δ-ALA-D) como proteína marcadora de intoxicação por metais e outras situações pró-oxidantes".

Aminolevulinate dehydratase (δ-ALA-D) as marker protein of intoxication with metals and other pro-oxidant situations

João B. T. Rocha, Rogério A. Saraiva, Solange C. Garcia, Fernanda S. Gravina and Cristina W. Nogueira

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REVIEW

Aminolevulinate dehydratase (δ-ALA-D) as marker protein of intoxication with metals and other pro-oxidant situations

Joao B. T. Rocha,* Rogerio A. Saraiva, Solange C. Garcia, Fernanda S. Gravina and Cristina W. Nogueira*

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δ-ALA-D is a metalloenzyme that has 3 vicinal thiol/thiolate groups that coordinate with Zn(II). The proximity between the sulfhydryl groups renders δ-ALA-D extremely sensitive to oxidation by soft electrophiles, such as Pb(II), Hg(II), As(III) and organoseleno and organotellurium compounds. In fact, blood δ-ALA-D is a classical biomarker of lead exposure in humans. The inhibition of δ-ALA-D can increase the concentration of 5-aminolevulinate (δ-ALA), which is a pro-oxidant compound. δ-ALA can generate oxidative stress that can further increase δ-ALA-D inhibition. Recently, data have been obtained indicating that the δ-ALA-D could be a marker of oxidative stress in human pathologies. In summary, considering its high sensitivity to pro-oxidant situations, δ-ALA-D can be considered a universal marker of oxidative stress.

1. δ-Aminolevulinate dehydratase or porporphobilinogen synthase: an easily oxidizable Zn(II)-thiol enzyme

Porphobilinogen synthase (PBGS, E.C. 4.2.1.24) or aminolevulinate dehydratase (δ-ALA-D) is an enzyme with a widespread distribution in nature[1–5] that catalyses the asymmetric condensation of two aminolevulinic acid molecules (δ-aminolevulinate; Fig. 1) to form the monopyrrole, porphobilinogen (PBG; Fig. 1). In cells, monopyrroles are the precursors for the synthesis of tetrapyrroles. Tetrapyrroles, such as heme and chlorophyll, are essential for aerobic metabolism and carbon fixation.[1–9] Consequently, toxic agents or metabolites that disrupt or interfere with tetrapyrrole synthesis can have profound effects on cell metabolism.[10] Accordingly, genetic δ-ALA-D deficiency is associated with hepatic porphyria in humans which can be exacerbated or precipitated by lead intoxication.[11–13] Lead can also cause 5-aminolevulinic aciduria and porphyria, which are related to δ-ALA-D inhibition.[12] Recent results indicate that the δ-ALA-D interacts with proteasome and, therefore, could be a physiological modulator of proteasomal activity. Metals that interfere with the δ-ALA-D activity can also change the modulation of proteasome by the δ-ALA-D.[14–18] The toxicological importance of δ-ALA-D inhibition by different metals and by oxidative stress found in a myriad pathological conditions is presented in the next sections of this review.

Mammalian δ-ALA-D is an oligomeric Zn(II)-enzyme that is fully active as an octamer[1,2,5–9,19] (Fig. 2) and it exists as an equilibrium of high-activity octamers and low-activity hexamers.[1,2] Mammalian δ-ALA-D has two Zn(II) binding sites per octamer and Zn(II) of one of these sites (ZnB) participates in the catalysis as a Lewis acid[9,20,21] (Fig. 3).

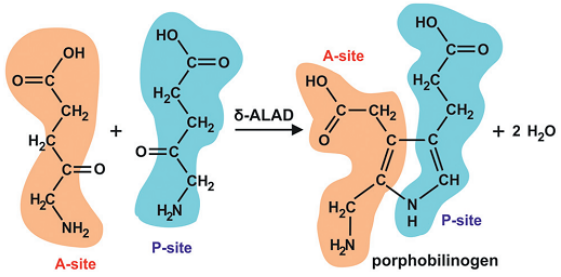

Fig. 1 Synthesis of porphobilinogen (PBG) from two 5-aminolevulinic acid (δ-ALA) molecules. Schematic illustration of the asymmetric condensation of two molecules of 5-aminolevulinic acid (δ-ALA) to form porphobilinogen (PBG). The A-site δ-ALA (light red) becomes the half of PBG that contains the acetyl moiety and the amino nitrogen while the P-site ALA (light blue) becomes the half of PBG that contains the propionyl moiety and the pyrrole nitrogen.

One special feature of the functional structure of δ-ALA-D is the presence of vicinal cysteinyl residues in its active site[23] (Fig. 3 and 4). These thiol groups are involved in the coordination of essential Zn(II) ions[21–23] (Fig. 3 and 4) and the proximity between them makes the enzyme particularly sensitive to oxidation.[23–25] Zn(II) is also involved in the stabilization of vicinal thiol/thiolate groups and its removal by chelating agents can accelerate enzyme autooxidation (Fig. 4).[26,27] Indeed, agents that oxidize –SH groups, metals with high affinity for thiol groups or that compete with the Zn(II) binding site can inhibit δ-ALA-D.[20,28–46]

Centro de Ciências Naturais e Exatas, Programa de Pós-Graduação em Bioquímica Toxicológica, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil. E-mail: jbtrocha@yahoo.com.br, criswn@quimica.ufsm.br; Tel: +55 55 3220 9462

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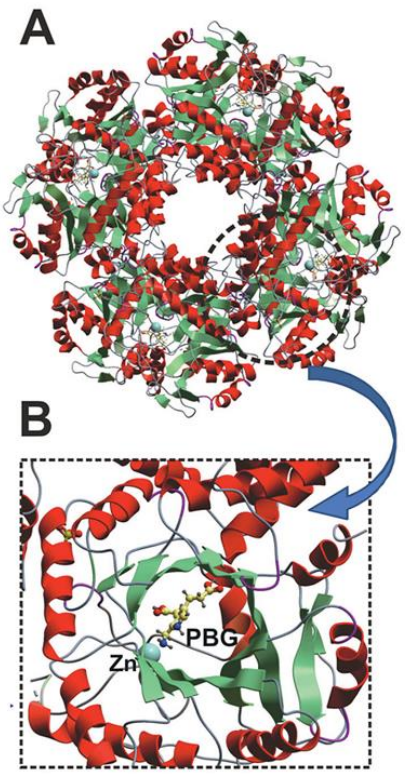

Fig. 2 Crystal structures of active octameric (A) conformation of human δ-ALA-D (PDB entry: 1E51). According to the crystal structure, each δ-ALA-D monomer (B) presents a catalytic zinc divalent ion (Zn) and the product porphobilinogen (PBG) inside the α/β barrel active center site. The β-strands are colored in green, α-helices in red and loops in gray or purple.

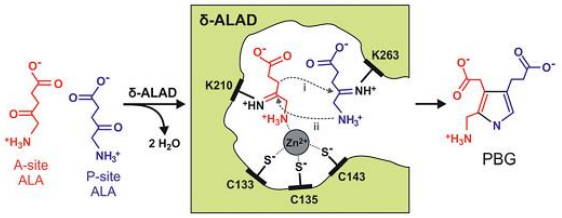

Fig. 3 Schematic illustration of the catalytic mechanism of δ-ALA-D. The figure is based on yeast δ-ALA-D crystal structures with PDB entries 1H7O and 1OHL with the asymmetric condensation of two δ-ALA (A-site δ-ALA, in red, and P-site, in blue) moieties through two Schiff-base linkages involving the positively charged lysine residues K210 (A-site δ-ALA) and K263 (P-site δ-ALA). Zn(II) is coordinated to the thiolate groups of C133, C135 and C143 and it plays an important role in substrate binding at the A-site (by linking to the amino nitrogen of PBG) and in stabilizing intermediates and transition structures during the enzymatic reaction.

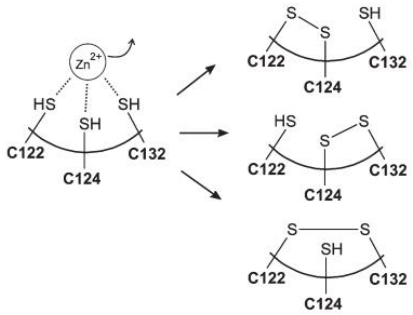

Fig. 4 Stabilization of thiol groups of δ-ALA-D by Zn(II). Removal of Zn(II) from the thiol-rich region ZnB of δ-ALA-D facilitates the oxidation of the enzyme and blocks catalysis. The preferential internal disulfide bridge after Zn(II) loss has not yet been clearly identified (three possibilities are shown in the figure).

2. δ-ALA-D as a marker of metal intoxication

For a long time, blood δ-ALA-D quantification has been considered an important clinical biomarker of Pb(II) exposure. Indeed, blood δ-ALA-D can be considered an early and reliable marker of Pb(II) poisoning in workers occupationally exposed to lead.[46–51] However, the modulation of δ-ALA-D by other metals, such as Hg(II), Cu(II), or Ag(II), indicates that its use to diagnose lead poisoning can be rather unspecific from the simultaneous intoxication with other metals.[52] Recent results suggest that the δ-ALA-D can also be "a molecular target of pro-oxidant situations", including here those associated with chronic-degenerative human diseases and in animal models of such diseases (details in Section 4). Thus, in addition to being a target of toxicants or metals that can directly oxidize or interact reversibly with thiol groups, the δ-ALA-D can be inhibited by an imbalance between reductive and oxidative metabolism found in different diseases.[53–55]

Of toxicological significance, the studies of Bechara and colleagues have demonstrated that the substrate of δ-ALA-D, the 5-aminolevulinic acid or δ-ALA (Fig. 1 and 3) can exhibit pro-oxidant properties under physiologically relevant conditions.[56–70] They have also demonstrated that markers of oxidative stress were altered in workers exposed to lead and postulated that this could be related to an increase in circulating 5-aminolevulinic acid (δ-ALA).[61] The inhibition of δ-ALA-D in lead poisoning[46–51] highlights the importance of its substrate, δ-ALA, on the molecular pathology of lead intoxication. Consequently, inhibition of δ-ALA-D by toxic agents or pathological conditions associated with oxidative stress can initiate a pro-oxidative vicious cycle that will further inhibit the δ-ALA-D and increase the concentration of potentially toxic compounds, such as δ-ALA and related metabolites.[70] Supporting this hypothesis, treatment of rats with δ-ALA induced cerebral oxidative stress and δ-ALA-D inhibition. Melatonin, which has antioxidant properties, blunted the pro-oxidant effects of δ-ALA and restored δ-ALA-D to normal levels.[71]

As mentioned above, δ-ALA-D has been utilized as a clinical indicator of lead intoxication because the enzyme is very sensitive to *in vitro* and *in vivo* Pb(II) inhibition.[72–87] However, the strong reactivity *in vitro* of the vicinal thiol groups of δ-ALA-D with soft electrophiles, such as Hg(II), Cd(II), Sn(II), As(III),

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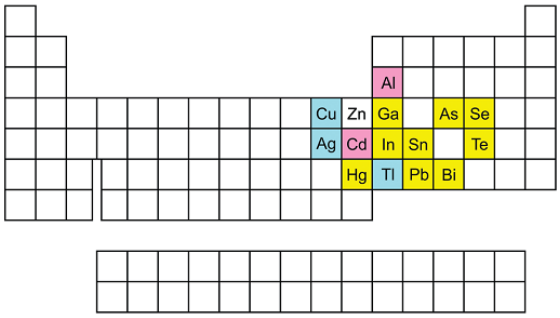

Fig. 5 Representation of elements from the periodic table that inhibit or stimulate δ-ALA-D activity. Zn(II) is the natural ligand of δ-ALA-D. Cd(II) and Al(III) can be either inhibitors or activators of the enzyme activity; whereas Ga(III), In(III), Hg(II), RHg(II), Sn(II), Pb(II), As(III), Bi(III), Se(IV), RSe-SeR, Te(IV) and RTe-TeR (where R can be an alkyl or aryl group) are either *in vitro* and/or *in vivo* inhibitor of δ-ALA-D. Pink indicates cations that can either stimulate or inhibit δ-ALA-D activity. Blue indicates cations that have been reported to inhibit mammalian δ-ALA-D activity *in vitro*. Yellow indicates those that inhibit δ-ALA-D activity either *in vitro* or *in vivo*.

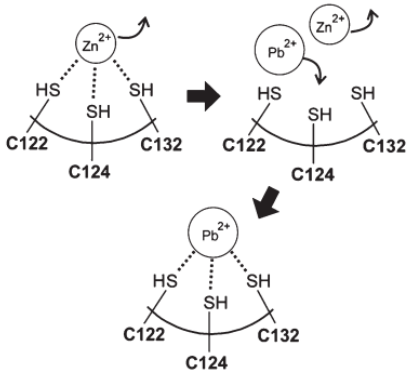

Fig. 6 Pb(II) Competition with Zn(II) at the active site of δ-ALA-D. δ-ALA-D inhibition by Pb(II) is mediated by substitution of Zn(II) from the thiolate-rich region in the mammalian enzyme.[20]

Bi(III), In(III), Tl(III), Se(IV) and Te(IV) indicated that any electrophile could be a potential *in vitro* or *in vivo* molecular inhibitor of mammalian δ-ALA-D (Fig. 5). The *in vitro* and *in vivo* inhibition of δ-ALA-D by these soft electrophiles is presented in the following sections. Most importantly, the combined exposure to Pb(II), As(III) and Cd(II) can have interactive toxic effects increasing or decreasing δ-ALA-D inhibition, depending on the duration of exposure and the tissue considered.[76] This elegant study highlights a crucial aspect that has been neglected and indicates the necessity of additional investigations to determine how these soft electrophiles interact to change or enhance their toxicological properties.

2.1. Pb(II) *in vitro*

Lead is a classical inhibitor of δ-ALA-D *in vitro* and Zn(II) can prevent or reverse the inhibition caused by Pb(II).[42,44,72–76]

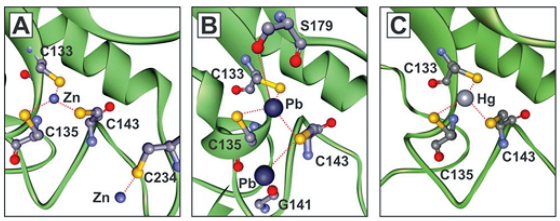

Fig. 7 Crystal structures of δ-ALA-D complexed with Zn(II) (A); Pb(II) (B) and Hg(II). Data are from yeast δ-ALA-D (A, PDB entry 1AW5), Pb(II) (B, PDB entry 1QNV) and Hg(II) (C, PDB entry 1QML). Pb(II) and Hg(II) can replace the enzyme's catalytic Zn(II) bound by three conserved cysteine side chains (C133, C135 and C143). Red dotted lines denote metal interactions (with distances ≤ 3.5 Å). The metal ions are shown in gray or blue scalled spheres. All the amino acid residues which are involved in molecular metal interaction are shown in ball-and-stick drawings. For clarity, hydrogen atoms are hidden. Atoms in sticks are colored as follows: carbon gray, oxygen red, nitrogen dark blue and sulfur yellow.

Treatment with Zn(II) *in vitro* can restore the δ-ALA-D activity following its inhibition caused by exposure to Pb(II) or Sn(II) *in vivo*.[74] Similar results were obtained in blood of humans exposed to lead. The inhibitory action of Pb(II) is related to displacement of Zn(II) from the active site of the mammalian δ-ALA-D, rather than to a direct oxidation of the enzyme's thiol groups by Pb(II)[20] (Fig. 6). Pb(II) is bulkier than Zn(II) and it, probably, cannot properly interact with amino acid residues and the substrate in the active centre of δ-ALA-D as does Zn(II) (Fig. 7A, B). Recently analysis based on molecular and computational models demonstrated that secondary bonding interactions (SBIs) and lone pair interactions profoundly influence the coordination geometry and binding affinity of Pb(II) and As(III) to peptides that simulate the environment of enzymes containing 3 thiol groups in close proximity.[88] Similar phenomena could occur at the Zn(II)B binding site of δ-ALA-D.

2.2. Pb(II) *in vivo*

Lead is a widespread toxicant that causes a variety of toxic effects in adults[89] and is extremely neurotoxic to the developing brain[90] causing long-lasting learning deficits in children.[91] The molecular mechanisms triggering lead toxicity involve the disruption of a variety of target proteins,[90–94] including δ-ALA-D. In fact, δ-ALA-D is an important blood lead binding protein[94] and it has been demonstrated that in erythrocytes δ-ALA-D is the primary target of Pb(II) instead of hemoglobin.[94] In contrast to blood, little is known about the relative affinity and binding of Pb(II) to the δ-ALA-D in comparison to other target proteins in soft tissues of mammals. The lack of knowledge on this subject is related to technical limitations since soft tissues contain several proteins that can bind Pb(II) and accurate information about the concentration of these targets is not available. However, from a toxicological point of view, it would be important to determine the *in vitro* affinity of Pb(II) to known target proteins to give support to computational studies, which could give us a rough calculation of Pb(II) distribution within living

cells. Such demanding and interdisciplinary studies could fill many gaps remaining in our knowledge about the interaction of Pb(II) and other toxic metals with specific target proteins.

The inhibition of δ-ALA-D by lead *in vivo* can be reversed by addition of Zn(II) and/or dithiothreitol (DTT) *in vitro*, indicating that the binding of Pb(II) *in vivo* is reversible. However, the strength of that inhibition is sufficient to trigger an increase in δ-ALA-D expression after exposure of rats to Pb(II).[95–97] The stimulation of δ-ALA-D synthesis after Pb(II) intoxication was interpreted as a compensatory response to the enzyme inhibition. We have observed that a short-term exposure of suckling rats to high doses of Pb(II) caused an increase in blood δ-ALA-D activity[35] and an increase in the reactivation index by dithiothreitol (DTT) + Zn(II) of two times,[98] corroborating the findings of Fujita and collaborators.[95–97]

In contrast to rats, workers from battery manufacturing plants exposed to lead exhibited an increase in δ-ALA-D gene methylation and a decrease in δ-ALA-D transcription. Most importantly, the increase in δ-ALA-D gene methylation was associated with an increased risk of lead intoxication.[99] These discrepancies between rats and humans can be related to species, duration and levels of lead exposure. The limited number of studies on this subject clearly indicates the necessity of more detailed scrutiny, particularly in view of the fact that δ-ALA-D gene methylation seems to be associated with an increased risk of lead toxicity in humans.

Of particular toxicological significance, Pb(II) exposure can be associated with increased oxidative stress and decreased δ-ALA-D activity.[77,82–85] For instance, children with aplastic anemia or suffering from neurological disorders (such as cerebral palsy, seizures, and encephalopathy) exhibit higher levels of blood lead, elevated thiobarbituric reactive species (TBARS), and lower δ-ALA-D activity and GSH levels than healthy subjects, indicating that part of the pro-oxidant effect of lead can be mediated by δ-ALA-D inhibition.[86,87] Similar results were obtained in urban adolescents with blood levels above 10 μg dL^{-1} [80] and in lead-exposed battery plant workers.[81]

The now well-established role of oxidative stress in Pb(II) intoxication highlights the early proposals by Bechara and colleagues[56–70] and strongly suggests that δ-ALA-D inhibition contributes to Pb(II) toxicity by either disrupting the heme biosynthesis pathway and/or by increasing the concentration of the potential pro-oxidant δ-ALA.

2.3. Cd(II) *in vitro*

Cadmium (Cd) is extremely toxic to living cells and an important environmental and occupational pollutant that can promote human diseases (*e.g.*, cancer and renal diseases).[100–103] The molecular mechanism of Cd(II) toxicity is not completely understood but it may involve disruption of Zn(II) homeostasis,[104] which can in turn modify the activity of Zn(II) enzymes, such as the mammalian δ-ALA-D.[105] Furthermore, Cd(II) can also stimulate the production of reactive oxygen species (ROS), either directly or indirectly *via* inhibition of antioxidant activities.[101,103] Accordingly, Cd(II)-induced inhibition of testicular δ-ALA-D and increase in oxidative stress are reversed by the antioxidant ebselen.[106]

In contrast to Pb(II), Cd(II) can replace Zn(II) in the active site of mammalian δ-ALA-D and promote the catalysis of PBG synthesis *in vitro*.[75,104–109] At neutral pH, the kinetic properties of mammalian Cd(II)-ALA-D are similar to those observed with the natural ligand Zn(II).[108] Davis and Avram[75] demonstrated that Cd(II) is a more potent activator of human erythrocytic δ-ALA-D and reverses Pb(II)-induced enzyme inhibition more efficiently than Zn(II). However, at high concentrations Cd(II) inhibits δ-ALA-D and Zn(II) cannot re-activate Cd(II)-inhibited δ-ALA-D.[75] This possibly indicates that at low concentrations Cd(II) binds to a stimulatory Zn(II) site without oxidizing or disrupting the thiol/thiolate interaction with the surrounding environment. The inhibitory effect of Cd(II) on δ-ALA-D at high concentrations can be related to its higher softness and to its stronger affinity for sulfhydryl groups than Zn(II). At low concentrations Cd(II) could replace Zn(II) and participate as Lewis acid in the catalysis, contributing to properly ionizing essential amino acid residues and substrates in the active site of the enzyme. On the other hand, at high concentrations Cd(II) could bind strongly and oxidize essential –SH group(s) involved in the coordination with Zn(II), or could oxidize –SH group(s) important for the maintenance of the native tertiary/quaternary structure of δ-ALA-D. However, the exact location of Cd(II) binding in the active centre of δ-ALA-D has not been precisely demonstrated yet and, in analogy to Pb(II), it is possible that Cd(II) can interact with the ZnA or ZnB sites of the enzyme (Fig. 8). Cd(II) can also form redox active complexes with dithiols and inhibit the mammalian ALA-D more efficiently than free Cd(II).[110]

2.4. Cd(II) *in vivo*

In contrast to *in vitro* data, which show that at low concentrations Cd(II) activates and at high concentrations it inhibits the δ-ALA-D, the results obtained following *in vivo* exposure are not homogeneous, probably reflecting the dose, duration of exposure and the source of the enzyme. Occupational exposure to Cd(II) has been associated with either no alterations in erythrocytic δ-ALA-D[46,111] or with a positive correlation between blood Cd levels and δ-ALA-D reactivation index,[112] indicating that exposure to Cd(II) can be associated with oxidation of δ-ALA-D. However, the subjects enrolled in the last study were also

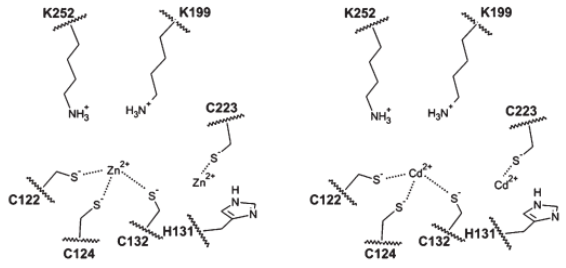

Fig. 8 Binding of Cd(II) to the Zn(II) binding sites in δ-ALA-D. At low concentrations, Cd(II) is expected to binding to the ZnB site (C122, C124 and C132) and to substitute Zn(II) and maintain δ-ALA-D activity. At high concentrations, Cd(II) possibly oxidizes these residues or binds to additional Zn(II) binding site (ZnA) represented by C223 (adapted from ref. 20).

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exposed to lead and the increase in the reactivation index could be the result of a complex interaction between Cd(II) and Pb(II) or the effect of the prevalence of Pb(II) over Cd(II).

Results in the literature show that Cd(II) can cause either no modification in rodent δ-ALA-D[37,113,114] or increase hepatic δ-ALA-D activity.[114] The authors also demonstrated that Cd(II) decreases the inhibitory effect of Pb(II) on hepatic but not on renal enzyme *in vitro*. The stimulation of hepatic δ-ALA-D activity and the reduced sensitivity to Pb(II) *in vitro* were attributed to induction of synthesis of Zn,Cd-thioneins by Cd(II).[114] In results obtained by our group, we observed an increase of the hepatic δ-ALA-D after exposure of rats to Cd(II).[105] However, under our experimental conditions, the stimulation of the δ-ALA-D by Cd(II) was not associated with an increase in metallothionein synthesis, but rather with an increase of the Zn levels in the liver. Recently, Whittaker and colleagues[76] demonstrated that Cd(II) could either stimulate (blood) or inhibit the δ-ALA-D (blood and kidney), depending on the length of exposure. Briefly, *in vivo* exposure to Cd(II) can have inhibitory or stimulatory effect on the δ-ALA-D activity; depending possibly on the concentrations reached by Cd(II) in different target tissues. The *in vivo* stimulatory effect of Cd(II) may be mediated by indirect induction of metallothioneins expression [114] or Zn(II) redistribution.[105] Nonetheless, a direct stimulation of the δ-ALA-D by Cd(II) cannot be ruled out.

2.5. Hg(II) *in vitro*

Inorganic and organic mercury salts (Hg(II), CH_3Hg(II), CH_3CH_2Hg(II)) are powerful electrophiles and have a strong affinity for soft nucleophiles. Biologically speaking, they have a strong affinity for –SH groups from target proteins.[115–118] In fact, Hg(II) and CH_3Hg(II) can inhibit δ-ALA-D *in vitro*.[32,35,37,110] However, the inhibitory potency of Hg(II) or CH_3Hg(II) is lower than Pb(II).[32,35,119] Consequently, considering that Pb(II) has a lower affinity for –SH groups than mercurials,[120] other factors in addition to affinity for thiol are possibly more important in determining the inhibition of δ-ALA-D by these metals. For instance, steric conflict, secondary bond energy interactions and lone pair interaction can strongly influence the coordination geometry and binding affinity of metals with vicinal thiols in models peptides.[88]

Detailed structural analysis indicates that Hg(II) binds to the same region as do Zn(II) and Pb(II)[21] (Fig. 7C), therefore indicating that Hg(II) can bind to the same sulfhydryl groups involved in the coordination with Zn(II). However, in view of the stronger affinity of Hg(II) for –SH groups than Pb(II) and Zn(II),[120] Zn(II) is unable to reactivate the enzyme inhibited by Hg(II) *in vitro* as it reactivates the Pb(II)-inhibited δ-ALA-D.[72–75] Furthermore, the distances of Hg(II) to cysteinyl residues in δ-ALA-D crystal are shorter than that observed with Zn(II).[21] Consequently, since Hg(II) has a stronger affinity for thiol groups than Pb(II) and Zn(II), it is possible that it binds to 2 –SH groups instead of coordinating with 3 thiolates (Fig. 9).[120] The interaction and binding of soft metals, such as Hg(II) and Pb(II), to thiolate-rich binding site are complex and little explored.[120] In fact, factors such as the rate of metal complexation reactions with thiol-rich sites, the geometry of interactions and the competition of soft

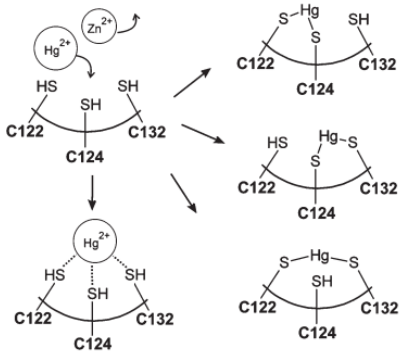

Fig. 9 Representation of the binding of Hg(II) to ZnB site in δ-ALA-D. Although Hg(II) can coordinate with 3 thiol groups in the δ-ALA-D active centre, its high affinity for –SH groups can determine a preferential binding to only 2 thiolates (four possibilities of Hg(II) binding to ZnB are presented).

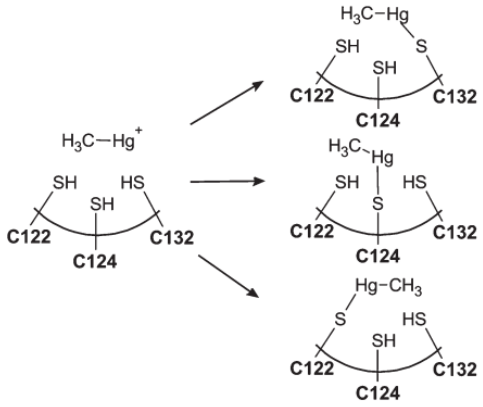

Fig. 10 Hypothetical interaction of MeHg (CH_3Hg(II)) with thiol groups from ZnB site in δ-ALA-D.

metals for the binding sites in aqueous solution have not yet been addressed in detail.[120] Consequently, our knowledge about the toxicity of metals will be greatly increased with studies addressing these important factors involved in the interaction of metals with thiol-rich proteins.

CH_3Hg(II) inhibits rodent δ-ALA-D *in vitro*;[32] however, there are no structural studies indicating the exact site of CH_3Hg(II) interaction with the δ-ALA-D. CH_3Hg(II) may interact with the same thiol groups in δ-ALA-D that does Hg(II). In low molecular weight compounds, CH_3Hg(II) preferentially binds to thiol in a 1 : 1 stoichiometry with a distance of about 2.4 Å.[121] Consequently, CH_3Hg(II) is expected to preferentially form complex(es) with a single or exceptionally with two –SH groups (Fig. 10). Indeed, in the case of the 3 cysteine residues of δ-ALA-D, the electronic structure of mercury ions (*e.g.* the fact that CH_3Hg(II) ion will likely bind to only one thiol group, as compared to 2 thiol groups in the case of Hg(II) ion) is the key factor that dictates the stronger affinity of these groups for Hg(II), when compared with CH_3Hg(II).[115] Thus, CH_3Hg(II) probably oxidizes one thiol group in δ-ALA-D; whereas Hg(II) oxidizes two or,

exceptionally, three groups in δ-ALA-D, either by forming a crosslink between two thiol groups (R1–S–Hg–S–R2) or a strong geometrical interaction with the three vicinal thiolate groups in the active center of δ-ALA-D[21] (Fig. 9 and 10). As discussed above, considering the important role of the geometry of metal binding to thiolate-rich region in proteins for their toxicity,[120] it will be important to determine in detail the $CH_3Hg(II)$ and Hg(II) binding motifs in δ-ALA-D. The determination of the potential bond lengths involved in the binding of these mercurials to δ-ALA-D will perhaps indicate that the distance between the outermost Cys residues is too long for some of the bonding motifs to be feasible. Thus, the expectation is that this type of studies will contribute to increase our understanding about the toxicity of these two forms of Hg and also of related toxic soft metals.

2.6. Hg(II) *in vivo*

$CH_3Hg(II)$ is an important ubiquitous environmental toxicant that can be found at high concentrations in piscivorous fish.[116–118,122,123] Consequently, the general population can be exposed to $CH_3Hg(II)$ *via* ingestion of contaminated fish. Of particular toxicological significance, there are two studies in the literature supporting the theory that the erythrocytic δ-ALA-D can be a target of $CH_3Hg(II)$. In a study carried out about 40 years ago, Schutz and Skerfving[124] reported that the δ-ALA-D activity in blood of subjects exposed to $CH_3Hg(II)$ *via* fish consumption was lower than that of non-exposed subjects and the inhibition was proportional to the total amount of Hg in the blood. Recently, negative correlations have been observed between the erythrocytic δ-ALA-D activity and Hg levels in the blood and hair of fish-eating subjects from the Amazonian region.[125] Antioxidant defense markers were negatively correlated with Hg levels, namely the antioxidant enzymes glutathione peroxidase and catalase. The authors also showed that the δ-ALA-D reactivation index was positively correlated with Hg levels and negatively correlated with antioxidant enzymes, supporting the notion that oxidative stress contributes to oxidizing the erythrocytic δ-ALA-D in Hg-exposed subjects. The study by Grotto *et al.* [125] illustrates that the inhibitory effect of $CH_3Hg(II)$ on δ-ALA-D activity can be mediated by a direct oxidation of thiol groups by $CH_3Hg(II)$ or indirectly *via* oxidative stress mediated oxidation of thiol groups.

Experimental studies indicate that short term exposure to high doses[32] or chronic exposure to low doses of $CH_3Hg(II)$ inhibit the δ-ALA-D and increase plasma MDA levels,[126] indicating a relationship between oxidative stress and δ-ALA-D inhibition. Here, it is important to point out that chronic $CH_3Hg(II)$ exposure have been reported to disrupt heme biosynthesis in rodents,[127,128] which may be related to δ-ALA-D inhibition.

Human exposure to inorganic mercury (Hg(0), Hg(I) or Hg(II)) can occur accidentally or occupationally under diverse situations. For instance, the use of skin-lightening creams with Hg has resulted in Hg poisoning, [129] or even the bizarre use of Hg for the treatment of head lice which caused fatal fulminate hepatic failure in a child.[130] The use of Hg(0) in dental amalgams and gold mining has been also associated with Hg exposure.[122,131,132] Furthermore, broken thermometers and compact fluorescent light bulbs can represent a domestic source of exposure to mercury.[122]

Hg(II) has a very high constant affinity for thiol groups.[115,121,133] However, there are no reports in the literature indicating that exposure to Hg(II) can inhibit human δ-ALA-D. In the study by Grotto *et al.*,[125] total Hg was determined in blood, plasma and hair. Since the subjects were from fish-eating communities, the mercury is probably derived from $CH_3Hg(II)$. Conversely, the Amazonian region has been exposed to high levels of Hg(0) used in gold mining.[132] Thus, without determining the speciation of Hg, we can speculate that the results of Grotto *et al.*[125] can be in part related to exposure to Hg(II) metabolically derived from Hg(0).

Exposure of mice and rats to high doses of Hg(II) inhibits the δ-ALA-D.[35–38] However, the sensitivity of the renal enzyme is greater than the hepatic δ-ALA-D, which in turn is greater than the enzyme from the brain.[35–38,134–136] These results are in agreement with the preferential distribution of Hg(II) to the kidney followed by the liver. Pre-exposure to Zn(II) decreases the inhibitory effect of Hg(II) on the rat renal and hepatic δ-ALA-D,[37,38] which is not related to changes in the distribution of Hg. The protection was related to metallothionein synthesis, which chelates Hg(II).

Hg(II) exposure has been reported to cause oxidative stress and inhibition of renal δ-ALA-D activity in rodents. Administration of the antioxidant lycopene blunted the pro-oxidant effect of Hg(II) and restored δ-ALA-D activity to control levels.[137] In contrast, astaxanthin, a carotenoid with antioxidant properties, prevented renal lipid peroxidation, but did not reverse Hg(II)-inhibited δ-ALA-D,[138] indicating a direct inhibitory effect of Hg(II) on the renal δ-ALA-D activity.

2.7. Al(III) *in vitro*

Aluminum is one the most abundant element on the earth's crust and it is largely utilized by humans in industry and household utensils. Furthermore, Al(III) is also employed in medicinal formulations, water clarification and as food additive.[139] Consequently, the daily exposure to aluminum can be high and the element has been implicated as a crucial factor in a variety of chronic disease.[139–141] Aluminum has also been indicated as the causative factor of a microcytic hypoproliferative anemia and it can worsen anemia in patients with end-stage renal disease. Even though the mechanism of aluminum-induced anemia is not completely understood, it seems to involve the disruption of heme biosynthesis.[142]

Al(III) has a low affinity for thiol groups.[34] However, there is at least one report showing that Al(III) inhibits the δ-ALAD activity *in vitro* with relatively high potency (in the low μmol L^{-1} rage).[143] In other studies, Al(III) has been shown to inhibit the δ-ALA-D only in the millimolar range.[144,145] In mouse blood, low concentrations of Al(III) were shown to stimulate the δ-ALA-D.[145] With purified hepatic δ-ALA-D, the inhibitory potency of Al(III) was also in the millimolar range and Zn(II) blunted the inhibitory effect of Al(III).[34] The reasons for such discrepancies are unknown, but they cannot be explained based only on the enzyme sources. Further studies need to be carried out to clarify these discrepancies.

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2.8. Al(III) *in vivo*

After *in vivo* exposure, Al(III) caused either no effect,[146,147] stimulation[144,148] or inhibition[144,149] of the δ-ALA-D activity, depending on the tissue, the chemical form of Al(III) administration (*e.g.* Al(III)-citrate or Al(III)) and the route of administration (oral or intraperitoneal).

2.9. Ga(III), In(III) and Tl(III) *in vitro* and *in vivo*

2.9.1. Ga(III). Gallium salts have gained importance as therapeutic agents for cancer treatment, in electronics as semiconductors and as a substitute for Hg in dental amalgam.[150,151] However, the toxicity of Ga(III) has not been completely understood yet. Gallium is located close to Zn in the periodic table (Fig. 5), which has motivated the study of Ga(III) as a potential inhibitor of the mammalian δ-ALA-D. In fact, Ga(III) is an *in vitro* and *in vivo* inhibitor of mammalian δ-ALA-D.[34,39,40] Exposure to GaAs has been reported to inhibit erythrocytic, brain, heart, renal and hepatic δ-ALA-D of rats.[40,152–159] Flora and colleagues demonstrated that GaAs causes inhibition of the δ-ALA-D and increases oxidative stress in rats. Under certain specific situations, antioxidant compounds blunted the toxic effects of GaAs.[155–159] Even though As(III) can also inhibit δ-ALA-D,[39] most of the inhibitory effect was attributed to Ga(III), because Ga(III) was about 40 to 200 times more potent than As(III) as an *in vitro* inhibitor of δ-ALAD. Additionally, the inhibition was partially prevented by Zn(II).[39]

2.9.2. In(III). In(III) is used as a semiconductor (as InAs). However, its toxicity has been so far only marginally studied.[160] In(III) (both as InAs or $InCl_3$ salts) inhibits δ-ALA-D, whereas arsenite (As(III)) is a much weaker *in vitro* inhibitor of δ-ALA-D than In(III).[41] Similarly, *in vivo* exposure of rats to In(III) (both as InAs and $InCl_3$) caused inhibition of the δ-ALA-D from different tissues. However, the inhibitory effect of As(III) was weaker than that of In(III) and depended on the tissue.[41]

2.9.3. Tl(III). Thallium is an extremely toxic element and it has caused accidental and occupational toxicity in humans. Little is known about the molecular mechanism(s) of Tl toxicity. Notably, Tl(III) has a high affinity for thiol groups and its toxicity can be associated with disruption of thiol-containing enzymes.[161] Al(III), Ga(III), and In(III) inhibit δ-ALA-D by competing with Zn(II), whereas Tl(III) and In(III) inhibit bovine δ-ALA-D by directly oxidizing essential sulfhydryl groups of enzyme.[34] In fact, the *in vitro* inhibitory effect of Tl(III) was reversed by DTT but not by Zn(II). In contrast to *in vitro* data, administration of Tl(III) to rats did not inhibit hepatic δ-ALA-D.[162]

2.10. As(III) *in vitro* and *in vivo*

Arsenic (As) is a widespread pollutant and its presence in water is a serious health hazard in several parts of the world.[163] Arsenic is thought to be involved in various adverse health effects, including cancer and neurodegenerative diseases.[164,165] However, the mechanism of arsenic toxicity is not completely known. As(III) can interact with thiol groups of proteins, disrupting their physiological roles. Accordingly, As(III) is a weak inhibitor of the rodent δ-ALA-D *in vitro*.[39,41] The molecular interaction of As(III) with δ-ALA-D has not yet been investigated. However, since As(III) and Pb(II) bind in a similar trigonal pyramidal coordination environments within peptidic frameworks using cysteinate ligands[88] and the δ-ALA-D has 3 vicinal thiol groups; it is possible that As(III) coordinate with thiolate groups of the enzyme with a similar geometry as does Pb(II). Corroboration of this inference can be found in studies that show that concomitant exposure to As(III) and Zn(II) *in vivo* protects the δ-ALA-D from As(III)-induced inhibition. On the other hand, post-treatment does not protect δ-ALA-D from As(III).[166,167]

There are several studies demonstrating that exposure of rodents to As(III) causes inhibition of the δ-ALA-D[39,41,76] and increases oxidative stress.[166–170] The administration of different types of antioxidant agents reversed the alterations induced by As(III).[171,172] However, quercitin did not restore the δ-ALA-D following the treatment with As(III), even though quercitin blunted the As(III)-induced oxidative stress.[173] This indicates that As(III) can directly oxidize the critical thiol groups of δ-ALA-D.

2.11. Sn(II) and Bi(III) *in vitro* and *in vivo*

2.11.1. Sn(II). Tin (Sn) in its trimethylated form is a ubiquitous environmental pollutant and it can cause a variety of toxic effects in living organisms,[174] including severe neurotoxic effects in mammals.[175] Furthermore, a possible role for toxic metals, including tin, has been suggested as an important factor involved in the initiation and progression of neurodegenerative diseases.[176] Sn(II) is also toxic to mammals and can cause oxidative stress and depletion of –SH groups.[177] Accordingly, Sn(II) administration caused inhibition of δ-ALA-D in rabbits, mice and rats,[178–182] which was prevented by Zn(II).[179] Although the mechanism of Sn(II)-induced inhibition of δ-ALA-D has not yet been thoroughly investigated, a study by Chiba and colleagues[181] strongly suggests that Sn(II) interacts in a more reversible way with the δ-ALA-D than Pb(II), since the blood enzyme activity returned to normal values more rapidly in Sn(II)- than in Pb(II)-intoxicated animals. Since Pb(II) competes with Zn(II) at ZnB site and Zn(II) can protect δ-ALA-D from the biochemical changes produced by Sn(II) administration,[179] we can hypothesize that Sn(II) inhibits δ-ALA-D at the same site of Pb(II).

2.11.2. Bi(III). Consumption of bismuth for different purposes is increasing and little is known about bismuth toxicity. The clinical utilization of bismuth iodoform paraffin paste has been associated with neurotoxic symptoms and chelation therapy with the dithiol, 2,3-dimercaptopropionic acid (DMPS) is effective in accelerating Bi excretion.[183] Accordingly, Bi(III) binds to thiol groups and can inhibit thiol-containing enzymes.[184] In fact, δ-ALA-D activity was inhibited after administration of Bi(III) to rats. However, the mechanism of such inhibition has not yet been comprehensively investigated.[185] Taking into account the reactivity of Bi(III) with thiol groups and its proximity to Pb(II) in the periodic table (Fig. 5), it is plausible to suppose that Bi(III) binds to the same region in the δ-ALA-D as does Pb(II). Bi(III) could coordinate with the 3 vicinal thiol groups located at the ZnB site in a geometry not exactly coincident with that of Pb(II) and that could partially explain the distinct toxicity of these

metals. Future computational and molecular studies are highly desirable to accurately determine the interaction of Bi(III) with the δ-ALA-D and other target proteins, particularly considering its increasing utilization in medicine and industry.

2.12. Children's exposure to metals

Few studies have investigated the effects of metals on δ-ALA-D activity in children, with the majority of studies focusing their attention on the effects of lead.[186–189] Children seem to be much more susceptible to lead intoxication as a consequence of their hand to mouth habit, increased gastrointestinal absorption and elevated respiratory rate.[189,190] Environmental pollution is the greatest source of child contamination with metals.[188] Children may be directly exposed to lead in dust, water and food.[191] Other sources of lead intoxication have been found in Mexican children, suggesting that the use of lead-glazed dishes and the habit of biting colored pencils increase lead levels in children.[191] The consequences of this exposure are deleterious to developing subjects, leading to a higher risk of neurological damage and cancer.[188] Moreover, δ-ALA-D activity has been shown to decrease after exposure to Pb, resulting in severe hematological consequences during development.[192] For instance, a recent study has shown that lead induced oxidative stress and decreased δ-ALA-D activity in children with aplastic anemia.[86] Thus, δ-ALA-D activity has been suggested to be a sensitive indicator of early hematological disorders related to lead exposure.[78]

Similarly to what happens in adults, children may also have variable susceptibility to lead intoxication[193] due to δ-ALA-D polymorphisms. Adult carriers of the ALAD-2 allele present higher blood lead levels and initially it was interpreted as they could be more susceptible to lead intoxication.[194] However, later on, it was found that δ-ALAD-2 has higher affinity and stability for lead than δ-ALAD-1.[195] Therefore, carriers of ALAD-2 allele may be less susceptible to intoxication, because of a putative protective effect of this polymorphism.[196] Child carriers of ALAD-2 allele also present significantly higher blood lead levels than ALAD-1 carriers.[197] Thus, they may also have a protective mechanism against Pb(II) toxicity. Interestingly, a study in Chile showed that children living in areas close to lead deposits are carriers of ALAD-2 allele more frequently than those living far away, suggesting a long-term selective defense associated with ALAD-2 polymorphism.[198]

The few studies cited above indicate that δ-ALA-D has not been sufficiently explored as a marker of metal exposure in children. Since children at risk of exposure are normally exposed to more than one toxic agent, it would be important to determine the activity of this enzyme in subjects at risk of exposure to different environmental toxicants.

3. δ-ALA-D as a marker of exposure to pro-oxidant compounds

3.1. Exposure to organochalcogens

Organochalcogen compounds are defined as the structures in which the chalcogen atom is directly bonded to a carbon (sp, sp^2 or sp^3) atom. In the following section the term organochalcogen will be used to refer specifically to organoselenium and organotellurium compounds.

Understanding of the molecular toxicity of organochalcogen compounds is still scarce in the literature. Much of the present knowledge of organochalcogen toxicity emerged from inorganic selenium research. Inorganic Se compounds can oxidize thiol groups and, in the case of selenium (Se(IV)), generate reactive oxygen species (ROS) during the catalytic oxidation of thiols.[199–202] These factors are believed to be the cause for toxicity of inorganic Se. To some extent, the interaction of organochalcogens with thiols has been reported to be similar to that associated with inorganic Se (for recent reviews see ref. 203–206 and references therein). The next paragraphs are intended to provide the reader with knowledge about the *in vitro* and *in vivo* inhibition of the δ-ALA-D by organochalcogens.

3.1.1. Exposure to organochalcogens *in vitro*. A study by Barbosa and collaborators in the late 1990s provided the initial observations that δ-ALA-D from different tissues of adult rats is a molecular target for the toxic effects of organochalcogens.[207] This study revealed that diphenyl diselenide, its analogues, as well as diphenyl ditelluride oxidize the δ-ALA-D.[207]

The importance of oxygen for the inhibitory effect of diphenyl diselenide and analogues was experimentally demonstrated and its removal from the medium decreased the inhibitory effect.[207] From these experiments evidence emerged that molecular oxygen can oxidize the selenol intermediates back to a diselenide (Fig. 11, panel A). The inhibition of the mammalian δ-ALA-D activity by diphenyl diselenide (and analogues) was dependent on the oxidation of cysteinyl residues located within the active site of the enzyme (Fig. 11, panel A).[207]

The presence of vicinal thiols in the active center of the mammalian,[208] fruit fly[209] and fish[210] δ-ALA-D indicates that they are crucial for the inhibitory action of diphenyl diselenide. This hypothesis was further supported by the fact that the δ-ALA-D from cucumber leaves is not sensitive to diphenyl diselenide (or its analogues).[24,25,207] In plants, the B site of δ-ALA-D is

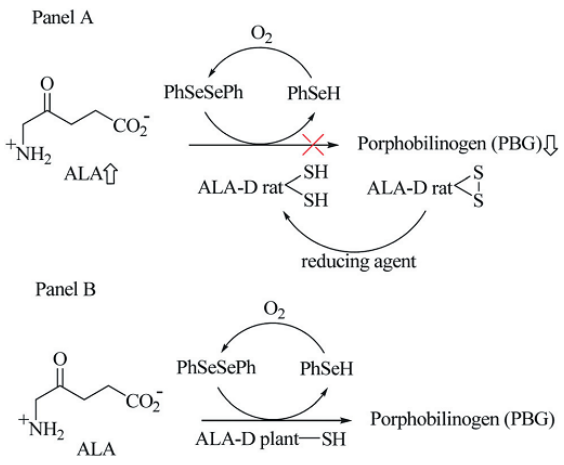

Fig. 11 Diphenyl diselenide inhibits δ-ALA-D activity from rats (panel A) but not from plants (panel B). The importance of vicinal thiol groups for inhibition of the rat enzyme.

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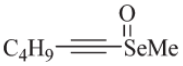
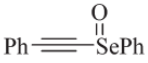
Table 1 Half maximal inhibitory concentration (IC_{50}) values for rat liver δ-ALA-D inhibition by selenides and their respective selenoxides

Compound	IC_{50} (μM)	Product[a]	IC_{50} (μM)	Ref.
PhSeMe	>400		104	212
C_4H_9—≡—SeMe	>400		72	212
Ph—≡—SePh	250		45	213

[a] Selenoxides were generated by reacting selenides with H_2O_2, for details on the experimental procedure the readers could refer tothe above cited references.

characterized by the substitution of some cysteinyl residues by acidic amino acids, which makes the enzyme less susceptible to oxidation (Fig. 11, panel B).[211]

The use of classical reducing agents, such as DTT, to restore δ-ALA-D activity reinforces the notion that organochalcogens inhibit the enzyme by oxidizing its essential cysteinyl residues (Fig. 11, panel A).[24,207,208] Despite the relatively poor thiol oxidant property of monoselenides, there is substantial evidence indicating that they can oxidize the δ-ALA-D after chemical or enzymatic transformation to their respective selenoxides.[212–215] Table 1 shows that selenoxides are more potent inhibitors of the rat liver δ-ALA-D than their respective parent compounds. This effect can be explained by the presence of selenium–oxygen double bond in which the pi (π) electron delocalization makes the selenium atom of selenoxides more electrophilic than that of selenides.

Taking into consideration the above mentioned and that the monooxygenation of selenides to their selenoxides by flavin-containing monooxygenases (FMO) has been reported *in vivo*,[214] δ-ALA-D could be a potential target of selenides toxicity after their oxidation to selenoxides by FMO.

Table 1 also shows that phenylselenoacetylene at high concentrations inhibits the δ-ALA-D activity from rat liver. This inhibitory effect was dependent on the conversion of phenylselenoacetylene to diphenyl diselenide.[215]

A number of organochalcogen compounds are inhibitors of the δ-ALA-D activity. Diselenides (Table 2, entries 1–4) are more effective inhibitors than the other organochalcogens shown in Table 2. The inhibitory potential of diphenyl diselenide varies depending on the source of enzyme (Table 2, entries 1, 7 and 18). The human erythrocytic δ-ALA-D is less sensitive to diphenyl diselenide than the rat liver or brain.

Diaryl diselenide substituted with chloro at the *para* position of the aromatic ring is the most effective inhibitor of the rat liver δ-ALA-D (Table 2, entry 2).

Despite the differences in the organic structure bonded to the selenium atom, carbohydrate diselenide derivatives were as potent as diphenyl diselenide in inhibiting δ-ALA-D from rat liver (Table 2, entries 1 and 4). By contrast, the IC_{50} values of diphenyl diselenide and its analogues were similar when the source of δ-ALA-D was rat brain, suggesting that the introduction of functional groups (trifluoromethyl, chloro or methoxyl) into the aromatic ring of diaryl diselenide does not alter its inhibitory effect (Table 2, entries 7–10). As seen on Table 2, selenofuranoses (entries 5–6)[216] and tris-selenides (entries 15–17)[217] are fair inhibitors of δ-ALA-D activity. It is also evident that δ-ALA-D from human erythrocytes is a potential target for diphenyl diselenide, ebselen and diphenyl ditelluride (Table 2, entries 18–20).[208]

The above mentioned studies provided clear evidence that δ-ALA-D from different sources can be a marker of organochalcogen exposure *in vitro*. Furthermore, δ-ALA-D inhibition may increase the concentration of 5-aminolevulinic acid (δ-ALA), which is a pro-oxidant molecule (Fig. 12).[68–70] Extrapolating the *in vitro* findings, the disruption of the aerobic metabolism by inhibiting the heme biosynthesis (Fig. 12) and the increase in the production of reactive oxygen species may be consequences of the δ-ALA-D inhibition by organochalcogens.

3.1.2. Exposure to organochalcogens: *in vitro vs. in vivo*. Although persuasive evidence has been found to suggest that δ-ALA-D activity is a marker of exposure to organochalcogens,[203–206] it is not clear whether the *in vitro* effects of organochalcogen compounds would be consistent with the *in vivo* outcomes. Amongst other reasons, the *in vivo* effect of certain compounds not only depend on the chemical form and the dose of the compound administered, but also on a variety of other factors such as the route of administration and the animal species.[203–206]

There is evidence suggesting that modifications of the organic moiety of organochalcogen compounds can have profound effects on their reactivity towards thiols. Accordingly, it has been demonstrated that diorganyl diselenide derived from cholesterol, dicholesteroyl diselenide, does not significantly inhibit the δ-ALA-D activity from different tissues after both *in vitro* or *in vivo* exposure.[220–222]

One of the first demonstrations that the effects of organochalcogens on the δ-ALA-D activity varied depending on the experimental conditions came from a study by Maciel and collaborators.[223] In that study, diphenyl diselenide inhibited the δ-ALA-D activity from brain, liver and kidney at a similar potency *in vitro*. However, when diphenyl diselenide was acutely or chronically administered to mice, the cerebral and hepatic δ-ALA-D activities were inhibited, but the renal enzyme was not altered.[223]

Diethyl 2-phenyl-2-tellurophenyl vinylphosphonate consistently inhibited the cerebral, renal and hepatic δ-ALA-D of mice *in vitro*. By contrast, exposure of mice for 12 days to this

Table 2 IC_{50} values for δ-ALA-D inhibition by different organochalcogen compounds[a]

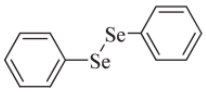
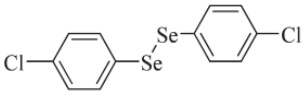
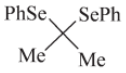
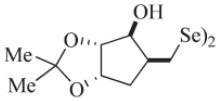
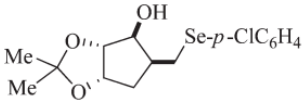
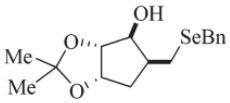
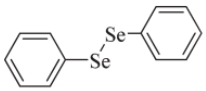
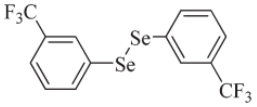
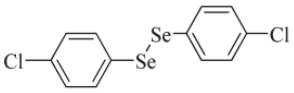
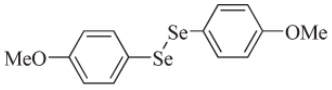
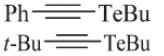
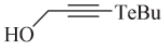
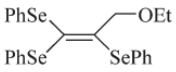
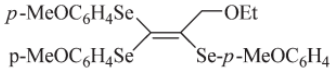
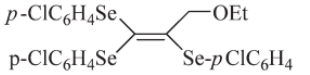
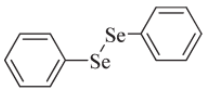
Entry	Compound	Enzyme source	IC_{50} (μM)	Ref.
1		Rat liver	9	207
2		Rat liver	2	207
3		Rat liver	9	207
4		Rat liver	10	216
5		Rat liver	>400	216
6		Rat liver	>200	216
7		Rat brain	4.6	217
8		Rat brain	6.6	217
9		Rat brain	4.3	217
10		Rat brain	4.3	217
11		Mouse brain	171	218
12		Mouse brain	177	218
13		Mouse brain	204	218
14		Mouse brain	213	218
15		Human erythrocyte	>200	219
16		Human erythrocyte	>200	219
17		Human erythrocyte	>400	219
18		Human erythrocyte	40	208

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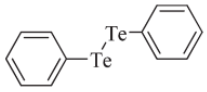
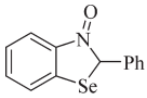
Table 2 *(Contd.)*

Entry	Compound	Enzyme source	IC_{50} (μM)	Ref.
19		Human erythrocyte	39	208
20		Human erythrocyte	100	208

[a] IC_{50} values were calculated using data obtained after pre-incubation of different concentrations of organochalcogens and the source of δ-ALA-D; for details of the experimental procedure readers should refer to the references cited above.

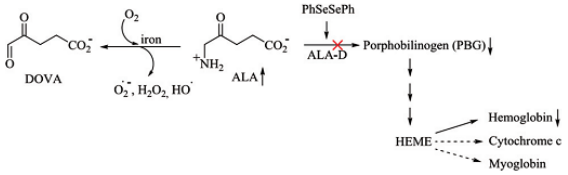


Fig. 12 The consequences of δ-ALA-D inhibition by diphenyl diselenide. The increase of ALA levels can produce reactive oxygen species and impairments in heme biosynthesis, which could disrupt aerobic metabolism.

organotellurium compound did not affect the δ-ALA-D from kidney, brain and liver.[224] The results indicated that the δ-ALA-D was not a molecular target for diethyl 2-phenyl-2-tellurophenyl vinylphosphonate *in vivo*, which can be explained by its weak ability to oxidize thiols.

As pointed out above, δ-ALA-D can be a marker of organochalcogen toxicity.[203–206] Consequently, the lack of inhibition of the enzyme by organochalcogens may indicate low toxicity of certain compounds. In this context, we have persuasive experimental results demonstrating that organochalcogens that do not inhibit the δ-ALA-D exhibit low toxicity *in vivo* (Table 3).

Moreover, organochalcogens are effective in reversing hepatic,[232–236] cerebral,[237] renal[238] and pulmonary[239] δ-ALA-D activity in experimental models in which this enzyme is inhibited by different xenobiotics. These results reinforcing the hypothesis that δ-ALA-D is not a target of organochalcogens at pharmacological doses.

3.1.3. Exposure to organochalcogens *in vivo*. δ-ALA-D has been postulated to be a useful biochemical marker for assessing organochalcogen toxicity. In fact, most experimental evidence now indicates that δ-ALA-D activity is inhibited by organochalcogens only at doses that caused toxicity in experimental animals. Table 4 shows that subchronic exposure to high doses of diphenyl diselenide or methyl phenyl selenide inhibited the hepatic δ-ALA-D activity and caused anemia in mice.[224,240–244] Thus, the δ-ALA-D inhibition following the exposure to high doses of organochalcogens may perturb the heme metabolic pathway, reducing the hemoglobin biosynthesis (Fig. 12).

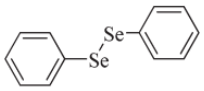
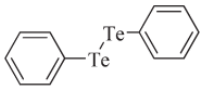
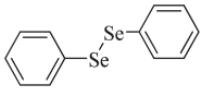
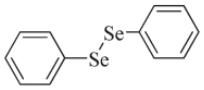
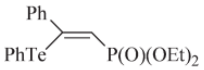
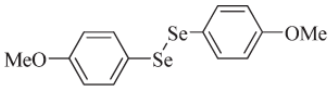
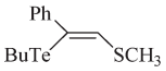
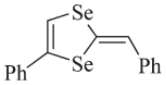
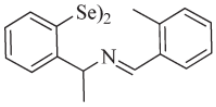
Diphenyl diselenide and its analogues, chloro and trifluoromethyl substituted diaryl diselenides, administered to rodents at doses near to the half maximal lethal dose (LD_{50}) inhibited δ-ALA-D from peripheral tissues and caused signs of systemic and hepatic toxicity (Table 4). Although 1-butyltellurenyl-2-methyl-thioheptene was administered at a single small dose to rats (compared to those used for Se compounds), the animals exhibited signs of systemic toxicity as demonstrated by the reduction of body weight gain, hepatotoxicity (increase of aspartate aminotransferase, AST, and alanine aminotransferase, ALT, activities), renotoxicity (increased urea levels) and dislipidemia (increased serum triglyceride levels) concurrent with the inhibition of δ-ALA-D activity.[244] Accordingly, organotellurium compounds have been reported to be more toxic agents to rodents than organoselenium compounds.[203,240]

As summarized in Table 5 rats exposed to a single high dose of ebselen or diphenyl ditelluride presented signs of renal (increased serum urea) and hepatic toxicity (increased serum AST and ALT) which were coincident with the inhibition of δ-ALA-D from erythrocytes. These observations further suggest that δ-ALA-D can be an enzymatic marker for organochalcogen toxicity.

The potential inhibition of δ-ALA-D activity may lead to neurotoxic effects. Evidence has been found showing that δ-ALA accumulation induces convulsions and that δ-ALA irreversibly inhibits glutamate uptake and is an antagonist of γ-aminobutyric acid (GABA) receptors.[245,246] As seen in Table 6, cerebral δ-ALA-D is inhibited by diphenyl diselenide at a dose that caused seizures in rat pups. On the other hand, diphenyl diselenide did not alter cerebral δ-ALA-D activity at anticonvulsant doses.[247–249] Accordingly, organoselenium compounds have been reported to have dual effects based on their dose-dependent contrasting action. At low doses, organoselenium has beneficial effects, whereas high doses are toxic. The threshold dose separating these opposing effects has not yet been precisely established.[203–206]

The consequences of lactational exposure to diphenyl ditelluride in the brains of suckling rats are shown in Table 6. Diphenyl ditelluride increased lipid peroxidation and inhibited catalase, superoxide dismutase and δ-ALA-D activities in cerebral structures of suckling rats.[250] As a consequence of δ-ALA-D inhibition, δ-ALA may undergo autooxidation thus facilitating the generation of reactive oxygen species. The ALA enoyl radical and 4,5-dioxovaleric acid (DOVA), the end products of δ-ALA oxidation, are reactive species that can disrupt cellular prooxidant/antioxidant balance (Fig. 12).[68–70] These *in vivo* observations generate a body of evidence reinforcing that δ-ALA-D

Table 3 Relationship between the absence of toxicity after organochalcogen exposure and the activity of δ-ALA-D in different species[a]

Compound	Animal species	Exposure	Signal of toxicity	Tissue	δ-ALA-D activity	Ref.
	Rat	8 weeks	None	Testes	Normal	225
	Rat	8 weeks	None	Testes	Normal	225
	Rabbit	8 months	None	Blood, hepatic, cerebral cortex	Increased	226, 227
	Rabbit	8 months	None	Renal, hippocampus	Normal	226, 227
	Mouse	12 days	None	Renal, hepatic, cerebral	Normal	224
	Mouse	72 hours	None	Liver, kidney, brain	Normal	228
	Rat	72 hours	None	Hepatic	Normal	229
	Mouse	1 hour	None	Brain	Normal	230
	Rat	3 days	None	Renal, hepatic, cerebral	Normal	231

[a] For details of the experimental exposure readers should refer to the references cited above.

from different sources can be an enzymatic marker of toxicity by organochalcogens.

4. δ-ALA-D as a marker of pro-oxidant situations

4.1. δ-ALA-D in animal models of human diseases

Reliable animal models of human diseases are essential for predicting applications in clinical research. In this regard, δ-ALA-D has been indicated as a useful screening marker for pathologies in the humans, mainly because δ-ALA-D activity can be often correlated with oxidative damage on proteins. Thus, it is essential to test its activity in classical animal models of human diseases to clarify whether it translates to real human diseases. Some classical models of diabetes type 1, such as alloxan- and streptozotozin (STZ)-induced diabetes, have already been characterized for δ-ALA-D activity. The results obtained with these drugs were similar to the findings observed in human type 1 diabetes patients in which an inhibition of δ-ALA-D was detected.[251–255] Investigating the molecular causes of δ-ALA-D inhibition, it was concluded that it could be caused by the glycation of the active lysine site or by the oxidation of cysteinyl residues of δ-ALA-D.[254] Indeed, it has been shown by the reactivation index (with DTT) that this enzyme is more oxidized in diabetic patients.[254] Accordingly, a recent work demonstrated that the administration of the antioxidant *N*-acetylcysteine to diabetic rats restored δ-ALA-D activity to control levels.[256]

Besides diabetes, δ-ALA-D activity has already been linked with a wide variety of other disorders and tested in different animal models of pathologies, such as obesity,[257] hypothyroidism,[258] hyperglycemia,[259] cancer,[260] paracetamol intoxication,[261] fatigue,[262,263] sepsis[264] among others.

Obesity and hyperglycemia are of great concern in our society, because they increase susceptibility to development of insulin resistance, diabetes, hypertension and additional complications.[265] Animal models of obesity and overweight, usually obtained by high fat diet feeding, have contributed to clarifying many issues related to these complications in the human body. Diet-induced hyperglycemia and high fat diet in animal models have been associated with increased ROS production and with reduced δ-ALA-D activity.[257,259] In fact, Folmer *et al.*[266] have demonstrated a strong negative correlation between oxidative

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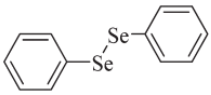
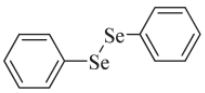
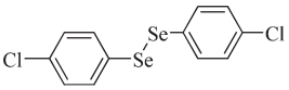
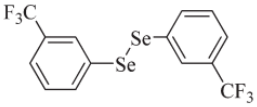
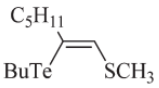
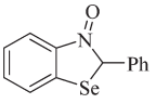
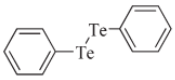
Table 4 Experimental evidence of δ-ALA-D from peripheral tissues as a marker of organochalcogen toxicity[a]

Compound	Exposure	Target tissue(s)	ALA-D activity	Signal of toxicity	Ref.
	250 μmol kg^{-1}, 14 days	Liver	Inhibited	Anemia in mice	223, 241
PhSeMe	500 μmol kg^{-1}, 30 days	Liver	Inhibited	Anemia in mice	242
	300 μmol kg^{-1}, 14 days	Kidney, liver	Inhibited	↓Body weight gain, ↑AST, ALT in rats	243
	380 μmol kg^{-1}, one dose	Liver, spleen	Inhibited	↓Body weight gain, ↓food and water intake in mice	228
	300 μmol kg^{-1}, one dose	Liver, spleen, kidney, brain	Inhibited	↓Body weight gain, ↓food and water intake in mice	228
	75 μmol kg^{-1}, one dose	Liver, spleen	Inhibited	↓Body weight gain,↑AST, ALT, urea and TG in rats	244

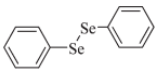
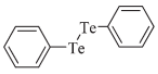
[a] For details of the experimental exposure readers should refer to the references cited above. AST – aspartate aminotransferase, ALT – alanine aminotransferase, TG – triglyceride, CAT – catalase, SOD – superoxide dismutase.

Table 5 Experimental evidence erythrocytes δ-ALA-D as a marker of organochalcogens toxicity in rats[a]

Compound	Exposure	Target tissue	δ-ALA-D activity	End point of toxicity
	340 μmol kg^{-1}, one dose	Erythrocyte	Inhibited	↑ Urea
	150 μmol kg^{-1}, one dose	Erythrocyte	Inhibited	↑ AST, ALT

[a] All data were collected from ref. 240. AST – aspartateaminotransferase, ALT – alanineaminotransferase.

Table 6 Experimental evidence of cerebral δ-ALA-D as a marker of organochalcogen toxicity in young rats[a]

Compound	Exposure	Target tissue(s)	δ-ALA-D activity	Signal of toxicity	Ref.
	50 mg kg^{-1}, one dose	Brain, liver	Inhibited	Seizures	249
	0.03 mg kg^{-1}, 14 days	Hippocampus, striatum	Inhibited	↑Lipid peroxidation ↓ CAT, SOD	250

[a] For details of the experimental exposure readers should refer to the references cited above. CAT – catalase, SOD – superoxide dismutase.

stress and δ-ALA-D activity in mice indicating that δ-ALA-D can be an earlier marker of metabolic changes associated with obesity and related pathologies.

Thyroid dysfunction in animal models (induced by propylthiouracil administration) was associated with inhibition of δ-ALA-D.[258] Moreover, hypothyroidism may be associated with oxidative stress.[267] However, δ-ALA-D activity was found to be higher in patients with hypothyroidism than in patients with normal thyroid function.[255] The discrepancies can be related to the time course of disease development (*i.e.*, after acute

induction in animal models *vs.* spontaneous and delayed development in humans), which could allow an adaptation to the metabolic alterations found in humans.

Laboratory animal models of human disorders have been quite helpful in clarifying the putative effects of oxidative stress and other metabolic changes on human δ-ALA-D; however, the number of studies is still limited, indicating that it would be crucial to test the δ-ALA-D activity in other models of human pathologies associated with oxidative stress to determine whether or not δ-ALA-D could be considered a universal marker of oxidative stress.

4.2. δ-ALA-D in human diseases and aging

δ-ALA-D activity is a potential biomarker for screenings of pathophysiological conditions, particularly in those associated with oxidative stress. Accordingly, blood δ-ALA-D has been reported to be inhibited in different pathological conditions, for instance, in hemodialysis patients,[268–271] after bone marrow transplantation,[272–274] in patients with cervical cancer[275] and in diabetes type 1 and 2.[251,254,255] Importantly, in various studies, a negative correlation was found between δ-ALA-D activity and oxidative stress. Human δ-ALA-D can also be inhibited after exposure to other pro-oxidant situations, for instance, after hyperoxygenation[276] and after exposure to a mixture of solvents in painters.[277] This is because δ-ALA-D contains vicinal -SH groups which can be easily oxidized[23–26] (Fig. 3 and 4), therefore, inhibiting its activity.[278]

Protein oxidation is a natural phenomenon that occurs in physiological and pathological processes, such as aging and age-associated conditions.[279] Aging can be defined either as a normal process of differentiation or as a progressive decrease in physiological function.[279] The free radical theory of aging[280] has gained experimental support with studies showing an exponential increase in oxidized proteins, lipids and DNA as a function of aging.[279] As aging and oxidative damage occurs, δ-ALA-D could be targeted by oxidative stress and be less active in the elderly. For instance, a study comparing young (8 weeks) and aged (38 weeks) mice demonstrated that in aged animals the δ-ALA-D activity was lower than in 8 week-old mice as a consequence of enzyme oxidation with aging.[257] Furthermore, δ-ALA-D activity has been suggested as a biomarker of oxidative stress in the elderly population.[281] In fact, δ-ALA-D activity decreased as a function of age and there was a negative correlation between age and blood δ-ALA-D activity between 60 to 84 years of age in elderly subjects.[281]

Age-associated pathologies have been demonstrated to contain altered forms of a variety of proteins, which could be related to protein oxidation and to a decrease in proteases involved in the degradation of aged proteins.[279] Protein oxidation is a common observation in age-related pathologies such as Parkinson's[282,283] and Alzheimer's diseases.[284–286] Interestingly, Alzheimer plaques contain amyloid-β protein, which binds to heme, potentially leading to heme deficiency.[287] Moreover, expression of aminolevulinate synthase (ALA-S) and porphobilinogen deaminase (PBG-D) mRNAs have been shown to be reduced in Alzheimer's disease, indicating that different points of heme biosynthesis can be altered in these chronic degenerative pathologies. Unfortunately, δ-ALA-D activity has not yet been tested in Alzheimer's or Parkinson's diseases. However, this enzyme could give important insights into the oxidative state of these age-associated conditions.[281]

5. Conclusions

δ-ALA-D has been considered a reliable and sensitive marker of Pb(II) exposure. However, in view of the presence of 3 vicinal thiol groups in its active centre, δ-ALA-D can also be oxidized by different soft electrophiles and by metals that compete with Zn(II) at its active centre. Recently, it has been postulated that δ-ALA-D could also be a molecular sensor of oxidative stress. In fact, the enzyme has been reported to be inhibited in experimental and in pathological situations associated with oxidative stress. Remarkably, the substrate of δ-ALA-D, 5-aminolevulinic acid (δ-ALA), is a pro-oxidant compound and δ-ALA-D inhibition can therefore indirectly increase oxidative stress *via* an increase in the levels of δ-ALA. The oxidative stress triggered by δ-ALA can further inhibit δ-ALA-D, thus setting forth a vicious toxic cycle. In short, δ-ALA-D is more than a biomarker of Pb(II). In fact, it can be considered a biomarker of exposure to different exogenous electrophiles and also to pro-oxidant situations found in relevant human pathologies. Thus, the pair δ-ALA and δ-ALA-D can be considered more than substrate and enzyme, because the inhibition of δ-ALA-D can increase δ-ALA levels that will further inhibit δ-ALA-D, contributing in this way to worsening the redox state of living cells.

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Artigo 2: “Estudos de *docking* molecular de disselenetos de diarila dissubstituídos como inibidores da enzima δ-aminolevulinato desidratase de mamíferos”

Molecular docking studies of disubstituted diaryl diselenides as mammalian δ-aminolevulinic acid dehydratase enzyme inhibitors

Rogério de Aquino Saraiva, Diones Caeran Bueno, Pablo Andrei Nogara e João Batista Teixeira da Rocha

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MOLECULAR DOCKING STUDIES OF DISUBSTITUTED DIARYL DISELENIDES AS MAMMALIAN δ-AMINOLEVULINIC ACID DEHYDRATASE ENZYME INHIBITORS

R. A. Saraiva, D. C. Bueno, P. A. Nogara, J. B. T. Rocha

Laboratório de Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus Universitário, Camobi, Santa Maria, RS, Brazil

δ-Aminolevulinic acid dehydratase (δ-ALAD) is a metalloprotein that catalyzes porphobilinogen formation. This enzyme is sensitive to pro-oxidants and classically used as a biomarker of lead (Pb) intoxication. Diphenyl diselenide [$(PhSe)_2$] and analogs bis(4-chlorophenyl) diselenide [$(pCl_3PhSe)_2$], bis(4-methoxyphenyl)diselenide [$(pCH_3OPhSe)_2$], and bis[3-(trifluoromethy)phenyl] diselenide [$(mCF_3PhSe)_2$] inhibit mammalian δ-ALAD by oxidizing enzyme cysteinyl residues, which are involved in diselenide-induced toxicity. 2-Cysteinyl residues from δ-ALAD are believed to sequentially interact with $(PhSe)_2$. Thus this study utilized protein–ligand docking analyses to determine which cysteinyl residues might be involved in the inhibitory effect of $(PhSe)_2$ and analogs toward δ-ALAD. All diselenides that interact in a similar manner with the active site of δ-ALAD were examined. Docking simulations indicated an important role for π–π interactions involving Phe208 and cation–π interactions involving Lys199 and Arg209 residues with the aromatic ring of $(PhSe)_2$ and analogs. Based upon these interactions an approximation between Se atoms and –SH of Cys124, with distances ranging between 3.3 Å and 3.5 Å, was obtained. These data support our previous postulations regarding the mechanism underlying δ-ALAD oxidation mediated by $(PhSe)_2$ and analogs. Based on protein–ligand docking analyses, data indicated that –SH of Cys124 attacks one of the Se atoms of –SH of $(PhSe)_2$ releasing one PhSeH (selenophenol). Subsequently, the –SH of Cys132 attacks the sulfur atom of Cys124 (from the bond of E-S-Se-Ph indermediate), generating the second $PhSe^-$, and the oxidized and inhibited δ-ALAD. In conclusion, AutoDock Vina 1.1.1 was a useful tool to search for diselenides inhibitors of δ-ALAD, and, most importantly, it provided insight into molecular mechanisms involved in enzyme inhibition.

Porphyrins are prosthetic groups particularly important in aerobic living systems since they are associated with a wide range of chemical properties, including oxygen binding, electron transfer, and light absorption (Mochizuki et al. 2010). The porphyrin structure consists basically of four modified pyrrole rings (tetrapyrroles), generally interconnected by methine bridges chelated to a centered divalent metal ion such as Fe^{2+}(in heme), Mg^{2+} (in chlorophyll), Ni^{2+} (in factor F_{430}), or Co^{2+} (in vitamin B_{12}) (Heinemann et al. 2008; Jaffe 2003). In the second step of porphyrin biosynthesis, the enzyme delta-aminolevulinic acid dehydratase (δ-ALAD) or porphobilinogen synthase catalyzes a unique asymmetric condensation of two molecules of δ-aminolevulinic acid (ALA) to form the monopyrrol precursor porphobilinogen (PBG) (Ajioka et al. 2006; Nogueira and Rocha 2011). The molecular

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Address correspondence to R. A. Saraiva, Laboratório de Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900, Santa Maria, RS, Brazil. E-mail: rogerioaqsaraiva@hotmail.com

mechanisms by which δ-ALAD from mammals catalyzes the formation of PBG is well-established from enzyme kinetic studies, site-directed mutagenesis, analysis of conformation and position of residues and substrate ALA in different crystal structures (Jaffe 2004), and recently, by density functional theory (DFT) studies (Erdtman et al. 2010). There is a consensus that the reaction involves covalent Schiff bases linkages between adjacent active site lysine residues (Lys199 and Lys252, at A (acetic) and P (propionic) site, respectively) and an interaction involving Zn^{2+} ion coordinated to the thiolates of three cysteinyl residues and the nitrogen from the $-NH_2$ group from the ALA substrate (A-site). Lys263 is essential in the catalysis because it is involved in the recognition of N from the pyrrole ring (P-site) from PBG (Erdtman et al. 2010).

In humans, δ-ALAD is encoded by a single gene located in chromosome 9q34, which has two co-dominant alleles, ALAD1 and ALAD2. The nature of this polymorphism is due to a G-to-C transversion in nucleotide 177, leading to a substitution of Asn59 (in ALA1) for Lys59 (in ALA2). Because lysine is a positive charged whereas asparagine is a neutral amino acid, the expression of these alleles results in three distinctly charged forms of the isozymes, designated ALAD1–1, ALAD1–2, and ALAD2–2. Thus, ALAD1–2 heterozygotes produce an enzyme that is more electronegative than that of ALAD1 homozygotes, and ALAD2 homozygotes produce an enzyme that is more electronegative than that of 1–2 heterozygotes (Fujihara et al. 2009; Kelada et al. 2001). Despite these differences in the total charge, all three isozymes display similar activities in PBG synthesis, since their active center site is not affected (Jaffe et al. 2001).

δ-ALAD has been used for a long time as a marker of lead (Pb) exposure (Oskarsson 1989; Takebayashi et al. 1993; Zhao et al. 2007). It appears that human δ-ALAD G177C polymorphism has been implicated in susceptibility to Pb-mediated toxicity due to differences in the electrical charge of the molecule resulting in ALAD2 with a higher affinity for Pb than ALAD1 (Fujihara et al. 2009; Kelada et al. 2001; Scinicariello et al. 2007). In addition, the mechanism underlying inhibition of Pb-induced effects involves a competition with Zn^{2+} coordinate with cysteinyl residues of the enzyme active site (Jaffe et al. 2001).

Recently, δ-ALAD was also suggested to serve as a potential bioindicator of oxidative stress (da Silva et al. 2007; Gonçalves et al. 2005; 2009; Grotto et al. 2010). Of particular toxicological significance, δ-ALAD inhibitors such as Pb, mercury (Hg), cadmium (Cd), beryllium (Be), succinilacetone, and 2,3-dimercaptopropanol are known to impair heme biosynthesis (Kusell et al. 1978; Rocha et al. 1993; Sakaguchi et al. 1997; Sassa and Kappas 1983) and increase ALA concentrations, which exert pro-oxidant activity under physiological conditions (Bechara et al. 1993).

Studies demonstrated that the simplest of the diaryl diselenides, diphenyl diselenide [$(PhSe)_2$] (Figure 1A), exhibits a variety of interesting biological effects, including antioxidant, antiulcer, neuroprotective, anti-inflammatory, antihyperglycemic, antiatherosclerotic and antihypercholesterolemic properties both in vivo and in vitro (de Bem et al. 2009; de Freitas et al. 2009; Hort et al. 2011; Nogueira and Rocha 2011; Sausen de Freitas et al. 2010). In contrast, $(PhSe)_2$ and analogs display cytotoxic properties (Posser et al. 2011) and disrupt the activity of thiol-containing proteins (Kade and Rocha 2010; Lugokenski et al. 2011; Puntel et al. 2010), including δ-ALAD from different sources (Barbosa et al. 1998; Farina et al. 2002; Golombieski et al. 2008). In agreement with these observations, in vivo exposure to $(PhSe)_2$ produced adverse effects (Ardais et al. 2010; Maciel et al. 2000; Straliotto et al. 2010) and inhibited mouse (*Mus musculus*), South American catfish (*Rhamdia quelen*), and fruit fly (*Drosophila melanogaster*) δ-ALAD by oxidation of sulfhydryl residues (Barbosa et al. 1998; Fachinetto et al. 2006; Farina et al. 2002; Golombieski et al. 2008; Soares et al. 2005).

In silico molecular docking has been widely employed as an important tool in order to assess interactions involving ligands and receptors (Roncaglioni and Benfenati 2008). This method combines mathematical models,

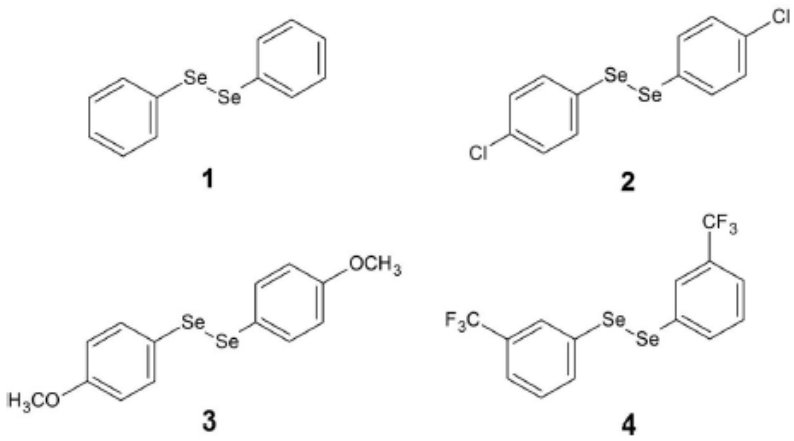

FIGURE 1. Chemical structure of disubstituted diaryl diselenides: 1, diphenyl diselenide $(PhSe)_2$; 2, bis(4-chlorophenyl) diselenide $(pCl_3PhSe)_2$; 3, bis (4-methoxyphenyl) diselenide $(pCH_3OPhSe)_2$; and 4, bis[3-(trifluoromethy)phenyl] diselenide $(mCF_3PhSe)_2$.

theoretical chemistry, quantum physics, and structural biochemistry (Kitchen et al. 2004). Utilizing this technique an understanding of the toxicological effects produced by the affinity of these ligands to a target enzyme can also be implemented (Sun and Yost 2007; Ma et al. 2011). Although studies demonstrated that diaryl diselenides inhibit δ-ALAD activity in vitro by sulfhydryl oxidation, no model has yet been proposed to explain this interaction. Previously, Farina et al. (2002) proposed that the inhibition of mammalian δ-ALAD may occur via oxidation of two vicinal –SH groups located at the active center of the enzyme (Figure 2). In this figure, a more reactive –SH group (labeled with *) undergoes a nucleophilic attack from one of the selenium (Se) atoms of diselenide (step 1), forming the first selenophenol (PhSeH). Sequentially, the vicinal less reactive –SH toward the –Se-Se bond is expected to attack the sulfur atom of the intermediate E–S*-SePh, forming the second selenophenol (PhSeH) and the oxidized and inhibited δ-ALAD (Figure 2). Thus, the aim of this study was to affirm that this mechanism underlying δ-ALAD inhibition by $(PhSe)_2$ occurred using in silico molecular models.

METHODS

Docking simulations of the diaryl diselenides with δ-ALAD were carried out using AutoDock Vina 1.1.1 (Trott and Olson 2010). The octameric crystal structure of human δ-ALAD obtained from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/) was used as macromolecule (PDB code 1E51). The diaryl diselenides $(PhSe)_2$, bis(4-chlorophenyl) diselenide $(pCl_3PhSe)_2$, bis(4-methoxyphenyl) diselenide $(pCH_3OPhSe)_2$, and bis[3-(trifluoromethy)phenyl] diselenide $(mCF_3PhSe)_2$ were constructed using the program Avogadro 0.9 and their geometry was optimized with the universal force field

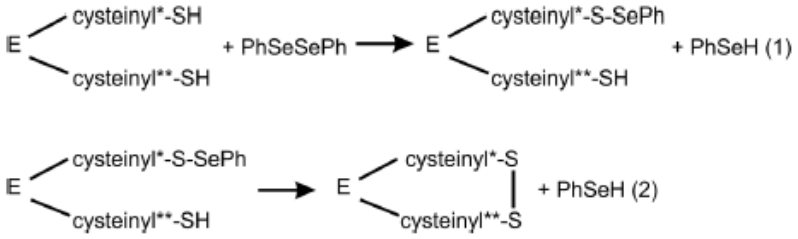

FIGURE 2. Proposed molecular mechanism of oxidation of catalytic thiols from δ-ALAD by $(PhSe)_2$. Adapted from Farina et al. (2002).

(UFF, which is a classical method of molecular optimization with a wide range of atomic parameters, including dependent atomic bond radii, a set of hybridization angles, van der Waals parameters, torsional and inversion barriers, and a set of effective nuclear charges) (Rappé et al. 1992). This was followed by the AM-1 method (a semiempirical method used to optimize the tridimensional structure of a molecule, according to atomic parameters previously calculated and obtained experimentally, in order to minimize energy and correct atom distances and dihedral angles) (Jakalian et al. 2002) from molecular orbital package (MOPAC), a set of semiempirical methods (Stewart 2009). Both ligands and macromolecule were previously prepared using AutoDock Tools (Morris et al. 2009). All rotatable bonds within the ligands were allowed to rotate freely, and the receptor was considered rigid. The grid was centered on the active site of $\alpha\beta$-barrel from δ-ALAD (x = 31.724, y = 71.555, and z = 58.081) and the dimensions of the grid box consisted of 20 Å × 26 Å × 20 Å points, with spacing of 1 Å. The exhaustiveness was set to 50. All other parameters were used as defaults. For each ligand docked, the conformation from the lowest binding free energy with inferred inhibitory reactivity was accepted as the best affinity model. The conformations and interactions were analyzed using the programs Accelrys Discovery Studio Visualizer 2.5 and PyMOL (Seeliger and DeGroot 2010).

RESULTS AND DISCUSSION

AutoDock Vina 1.1.1 is a recent docking program that operates by pairing an empirically weighted scoring function containing terms for values such as hydrogen bonding, hydrophobic interaction (van der Waals), rotatable bond penalties, and a sophisticated gradient-based local search as a global optimization algorithm (Chang et al. 2010; Trott and Olson 2010). In order to ensure the efficiency of AutoDock Vina program as a reliable model, a re-docking of the crystal ligand PBG in the active site of δ-ALAD was previously performed. The best

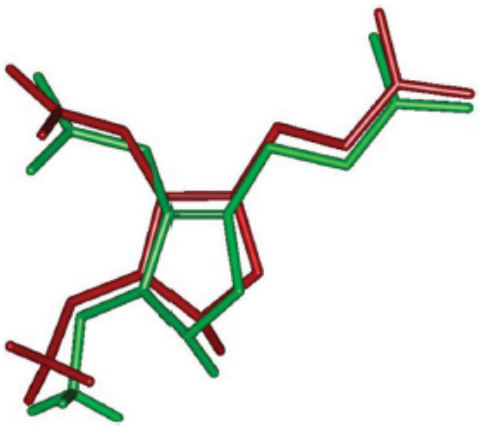

FIGURE 3. Molecular overlapping of the crystal ligand PBG (green) and the best pose of PBG proposed by AutoDock Vina 1.1 program (red), with RMSD = 1.074 Å (color figure available online).

conformation from the crystal ligand PBG proposed by docking in silico was almost identical with the original conformation, showing a root mean square deviation (RMSD) of 1.074Å (Figure 3). A RMSD value less than 2 Å is a criterion often used for the correct bound structure prediction (Bursulaya et al. 2003). Thus evidence indicates that AutoDock Vina can be reliably used in this study as a docking tool in the prediction of enzyme–ligand interactions.

Graphical representations of the docking results of $(PhSe)_2$ and analogs are illustrated in Figures 4 and 5. The molecular models proposed by virtual docking analyses suggest that all diaryl diselenides interact similarly with the active site of δ-ALAD (Figure 4).

The free energies of binding (ΔG_{bind}) of these interactions are given in Table 2. All values obtained are equal or lower than the ΔG_{bind} of the native δ-ALAD product ligand PBG (ranging from –6.0 to –5.4 kcal.mol^{-1}). Consequently, the binding of $(PhSe)_2$ and analogs to the active site of δ-ALAD is expected to be a spontaneous process. Furthermore, the interaction of $(PhSe)_2$ and analogs with the active center of δ-ALAD occurred via π–π stacking and cation–π and C–H . . . π interactions. Consequently, these types of interactions are fundamental for inhibition of δ-ALAD by aromatic diselenides. The π–π interaction was formed between the phenyl group of the diaryl diselenide and the aromatic

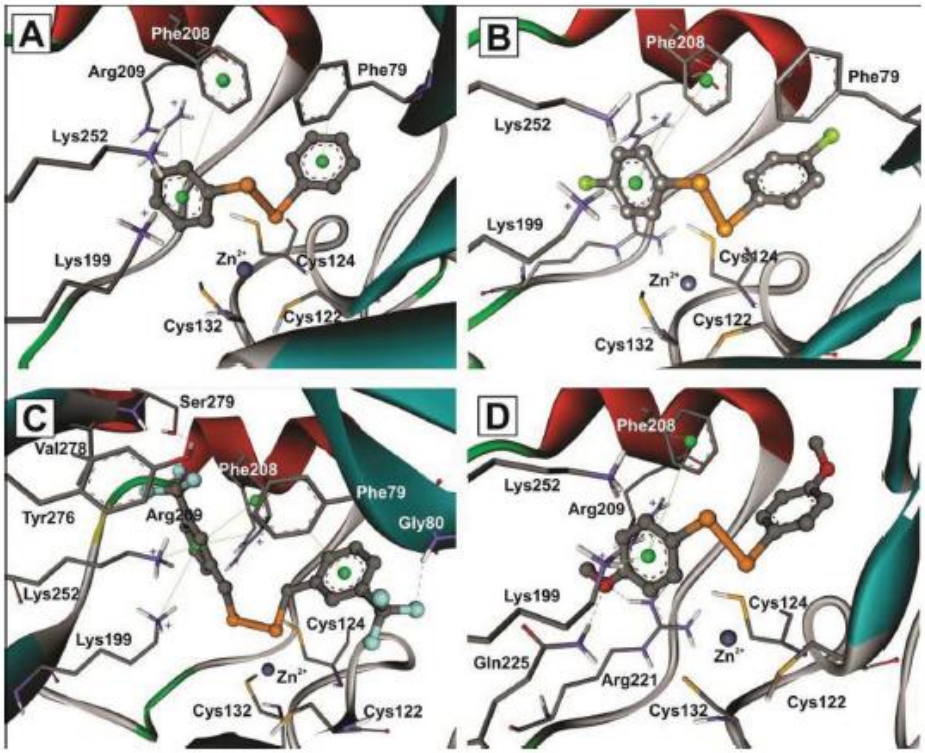

FIGURE 4. Representative molecular models of (A) diphenyl diselenide (yellow), (B) bis(4-chlorophenyl) diselenide, (C) bis[3-(trifluoromethy)phenyl] diselenide diphenyl diselenide, and (D) bis(4-methoxyphenyl) diselenide binding the active site of δ-ALAD. The green lines denote the C–H . . . π, π–π stacking, and cation–π interactions. The dark-blue dotted lines denote hydrogen bonds. The green ball inside the aromatic structure denotes its centroid. All the amino acid residues that are involved in molecular interaction are shown in the stick drawing. Ligands are shown in ball-and-stick drawing. Atoms in sticks are colored as follows: hydrogen white, carbon gray, oxygen red, nitrogen light blue, sulfur yellow, and selenium orange (color figure available online).

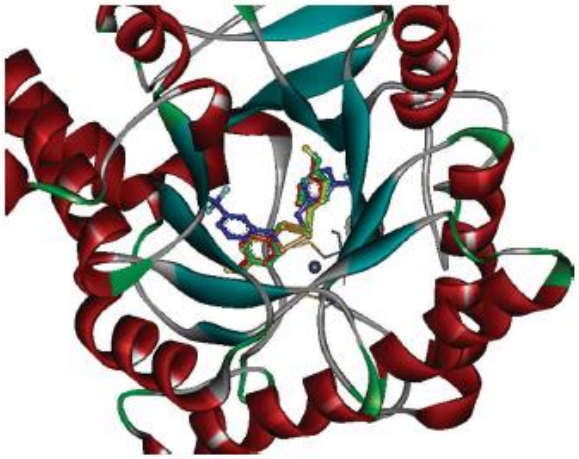

FIGURE 5. Molecular overlapping of the best pose of ligands diphenyl diselenide (yellow), bis(4-chlorophenyl) diselenide (green), bis(4-methoxyphenyl) diselenide (red), and bis[3-(trifluoromethy)phenyl] diselenide (blue) binding the $\alpha\beta$-barrel active site of human δ-ALAD (color figure available online).

ring of Phe208 from δ-ALAD. Cation–π interactions were observed between the phenyl group of the $(PhSe)_2$ and positively charged residues Lys199 (important for the formation of Schiff-base linkage with the substrate ALA "A-site") and Arg209 (Figure 4). C–H . . . π

interaction was noted between the aromatic $C_{\delta 2}$-H of Phe79 and one of the phenyl groups of the ligands (Figure 4). Hydrogen bonds between the oxygen from methoxyl group of $(pCH_3OPhSe)_2$ and the hydrogen from residues Gln225 and Arg221 (Figure 4D) and between two fluorine atoms from–CF_3 group of $(mCF_3PhSe)_2$ and the hydrogen from residues Tyr276 and Gly80 were also noted. Taken together, these interactions contribute to forming stable complexes between diaryl diselenides and the active center of δ-ALAD. Of particular importance for the mechanism underlying δ-ALAD- induced inhibition by diaryl diselenide compounds, these distinct types of interactions allow an approximation between the reactive electrophile moiety –Se–Se– from the ligands to the nucleophile –SH groups Cys122, Cys124, and Cys132 with distances ranging from 3.3 to 6.0Å (Figure 4 and Table 1). This orientation adopted by the ligands is of crucial importance to permit the oxidation of these cysteinyl residues located in close proximity in the catalytic center of δ-ALAD. The oxidation of these residues is expected to produce an immediate inhibition of enzyme, which is accompanied by release of Zn^{2+}, leading to a conformational state that is less susceptible to reactivation by reducing agents (Emanuelli et al. 1998).

Barbosa et al. (1998) and Farina et al. (2002) demonstrated that $(PhSe)_2$ inhibited animal but not plant δ-ALAD by thiol oxidation. Indeed, the active site of δ-ALAD in animals and plants is different. Although in animals the catalytic Zn^{2+} ion coordinates sulfur atoms from cysteine residues, in contrast, in plants, cysteine residues are substituted by aspartic acid residues and the metal Zn^{2+} is replaced by Mg^{2+}, which presents a significant preference for binding to oxygen atom from water and aspartic acid, serine, and glutamine residues (Jaffe 2003). These findings support the belief that $(PhSe)_2$ is not able to inhibit plant δ-ALAD due to significant differences in its active site, including the absence of cysteine residues.

Previous studies indicated that $(PhSe)_2$ and analogs inhibit hepatic, renal, and cerebral δ-ALAD in vitro (Barbosa et al. 1998; Farina et al. 2002). The first step of oxidation of –SH groups involves the reaction of the enzyme with diaryl diselenide to yield the intermediate E-Cys-S–SePh via an attack by the most reactive cysteinyl residue (indicate by asterisk in Figure 1) in one of the Se atoms of diaryl diselenide. Subsequently, the second more nucleophilic cysteinyl residue attacks the sulfur atom of the -S-Se- bond of the intermediate (E-Cys*-S–SePh) due to its close spatial proximity to the more reactive residue, producing the oxidized enzyme (E-Cys-S–S-Cys-E) and two molecules of selenophenol (Figure 6). Here, docking analyses indicated that the residue Cys124 is the nearest thiolate to Se atoms of $(PhSe)_2$ and analogs (see distances in Table 1). In addition, a positive correlation was found between the ΔG_{bind} and the distance between the nearest Se of diaryl diselenides to Cys124 and the sulfur atom of Cys124 (R^2 = .9958, Figure 7), suggesting that the approximation of the –SH group from Cys124 to one of the Se atoms

TABLE 1. Free Energy of Binding Proposed by AutoDock Vina and the Distance of Nearest Selenium Atom of Ligands to Sulfur From Thiol Group of Cys122, Cys124, and Cys132 Docked to Human δ-ALAD Enzyme

Ligand	Proposed free energy of binding (kcal/mol)	Distances from the nearest Se atom of ligands to S of cysteinyl residues (Å)		
		Cys122	Cys124	Cys132
PBG	–5.4	—	—	—
$(PhSe)_2$	–6.0	4.363	3.332	5.816
$(mCF_3PhSe)_2$	–5.7	4.102	3.399	5.513
$(pClPhSe)_2$	–5.8	4.317	3.384	5.637
$(pCH_3OPhSe)_2$	–5.4	4.376	3.466	5.689

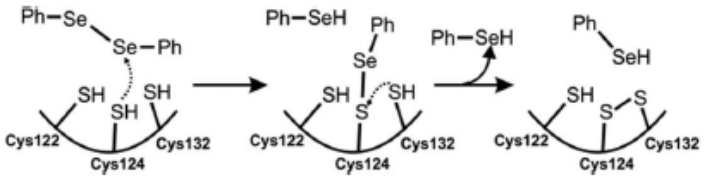

FIGURE 6. Additional proposed molecular mechanism of oxidation of catalytic thiols from human δ-ALAD by $(PhSe)_2$, according to docking molecular studies *in silico*.

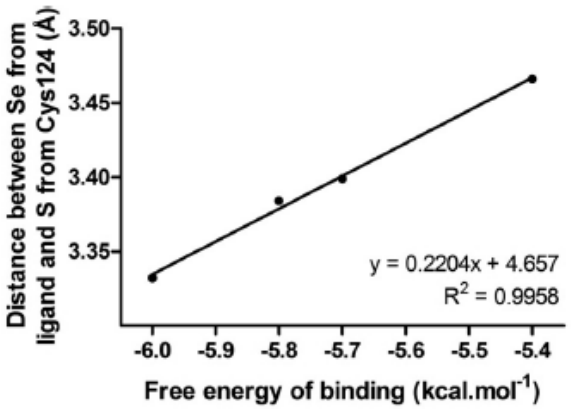

FIGURE 7. Correlation between the free energy of binding (ΔG_{bind}) predicted by AutoDock Vina program and the distance between the sulfur atom from Cys124 and the nearest selenium of $(PhSe)_2$ and analogs binding human δ-ALAD.

from diselenides may be crucial for $(PhSe)_2$ and analogs-mediated inhibition of δ-ALAD. Consequently, evidence indicates that the sulfur atom from Cys124 attacks the –Se-Se bond in diaryl diselenides in order to form the intermediate E-Cys124-S-SePh.

According to the distances of –SH from Cys122, Cys124 and Cys132 in human δ-ALAD crystal (Table 2), Cys132 presents the nearest thiol from thiol of Cys124. Consequently, it is postulated that Cys132 attacks the sulfur atom of the intermediate E–Cys124–S–SePh, forming the oxidized enzyme (–Cys124–S–S–Cys132–) and 2 PhSeH molecules (Figure 7).

TABLE 2. Distances Between Thiol Groups From Cysteinyl Residues of Catalytic Active Site of Human δ-ALAD Enzyme

Residues	Distance (Å)
–SH of Cys122 and –SH of Cys124	4.626
–SH of Cys122 and –SH of Cys132	4.423
–SH of Cys124 and –SH of Cys132	4.255

CONCLUSIONS

The molecular docking analyses performed here indicate that –Cys124 is probably the critical mammalian nucleophilic center, which initiates the thiolate attack on the electrophile –Se–Se– moiety of $(PhSe)_2$ and analogs, forming an intermediate of the type E–S–Se–Ph. This –S–Se– bond is subsequently attacked by the vicinal Cys132 –SH group, resulting in the formation of an internal disulfide bond between Cys124 and Cys132 in mammalian δ-ALAD. This is expected to produce inhibition of the enzyme and release of the Zn^{2+} ion (which was coordinated to the three thiolate ($-S^-$) groups of the δ-ALAD active center). The two molecules of PhSeH formed at the expense of cysteinyl residues oxidation are easily oxidized back to –Se–Se–[$(PhSe)_2$] by molecular oxygen. Data presented support a sequential interaction of two vicinal cysteinyl residues of mammalian δ-ALAD with $(PhSe)_2$. The selenol intermediate molecules formed (2PhSeH) react promptly with oxygen, regenerating $(PhSe)_2$. Consequently, the net result of the interaction of $(PhSe)_2$ with δ-ALAD is the inactivation of this enzyme by catalytic amounts of diselenides. In summary, AutoDock Vina 1.1.1 is a useful tool to search for diselenides inhibitors of δ-ALAD and, most importantly, it provides insights with respect to the molecular mechanisms involved in δ-ALAD-induced inhibition. In addition, the results presented here suggest that the rational search for inhibitors of enzymes needs to start with in silico docking analyses.

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3.2 Manuscrito: “Descrição por Bioquímica Quântica das energias de interação envolvendo o organocalcogênio disseleneto de difenila em complexo com a enzima δ-aminolevulinato desidratase”.

Quantum Biochemistry description of the interaction energies for the organochalcogen diphenyl diselenide in complex with δ-aminolevulinic acid dehydratase enzyme

Rogério A. Saraiva, Pablo A. Nogara, Roner F. Costa, Eveline M. Bezerra, Thiago H. Lugokenski, Everton W. Caetano; Irwin R. A. Menezes; Valder N. Freire, and João B. T. Rocha.

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A Quantum Biochemistry description of the interaction energies for the organochalcogen diphenyl diselenide in complex with δ-aminolevulinic acid dehydratase enzyme

Rogério A. Saraiva[1], Pablo A. Nogara[1], Roner F. Costa[2], Eveline M. Bezerra[2], Thiago H. Lugokenski[1], Everton W. Caetano[3]; Irwin R. Menezes[4], Valder N. Freire[2]; and João B. T. Rocha[1]*.

[1]Programa de Pós-Graduação em Bioquímica Toxicológica, Universidade Federal de Santa Maria (UFSM), 97105-900, Santa Maria, RS, Brazil.
[2]Departamento de Física, Universidade Federal do Ceará (UFC), 60455-760, Fortaleza, CE, Brazil.
[3]Instituto Federal de Educação, Ciência e Tecnologia do Ceará (IFCE), 60040-531, Fortaleza, CE, Brazil.
[4]Departamento de Química Biológica, Universidade Regional do Cariri (URCA), 63100-000, Crato, CE, Brazil.

Abstract

δ-aminolevulinic acid dehydratase (δ-ALA-D) is an essential metalloprotein involved in porphyrin biosynthesis. Consequently, its inhibition is implicated in a set of physiological disorders, including impairment of heme synthesis, neurotoxicity and oxidative stress. In the last years, the organochalcogen diphenyl diselenide [$(PhSe)_2$] has called attention as a novel promising drug with neuroprotective, antioxidant, anti-atherosclerotic and anti-inflammatory properties. Contrariwise, $(PhSe)_2$ can be toxic due, in part, to the ability to oxidize catalytic sulfhydryl groups from δ-ALA-D. In order to give important support to the rational design of novel diselenide drugs with low affinity for δ-ALA-D, we aimed to understand the inhibition mechanism of δ-ALA-D by $(PhSe)_2$ using *in silico* molecular modeling and theoretical quantum biochemistry calculations. For this purpose, interaction energies between $(PhSe)_2$ and neighboring residues at the active site of δ-ALA-D were calculated by Molecular Fractionation with Conjugated Caps (MFCC) method using the DFT approach. According to MFCC, interactions could occur up to 9 Å distance from the centroid of $(PhSe)_2$ active site. Phe208, Phe79, Cys122, Cys124, Pro125, Asp120, Lys199, Lys252 and Cys132 displayed strong attraction energy to $(PhSe)_2$. The representative molecular model is in accordance with *in vitro* assays and gives mechanistic support to previous speculative mechanism of

* Corresponding author: . Email: jbtrocha@yahoo.com.br, Tel. +55 55 3220 9462

inhibition. Phenyl moieties in $(PhSe)_2$ can be strongly attracted by aromatic and positive charged residues from δ-ALA-D active site. This allows the approximation of the reactive electrophile moiety Se-Se to the nucleophile $–S^-$ groups from Cys122, Cys124 and Cys132, facilitating the release of coordinated Zn(II), thiol oxidation and formation of 2 molecules of phenylselenol (PhSeH). In conclusion, the presence of aromatic moieties in $(PhSe)_2$ and its reactive electrophile moiety Se-Se are crucial to δ-ALA-D inhibition, which leads to thiol oxidation and consequent impairment of its activity.

1. Introduction

Porphyrins are essential biomolecules to all forms of life, since they play an important role as prosthetic groups or coenzymes and are involved in a wide range of physiological and biochemical processes, including pigmentation, energy production, electron transfer, gas transport and light absorption (Mochizuki et al., 2010). Structurally, they are composed of four modified pyrrole rings generally interconnected by methinic bridges (=CH), and a central region with a bivalent metal ion (e. g. Fe(II) in heme, Mg(II) in chlorophyll and Co(II) in vitamin B_{12}) (Heinemann et al., 2008; Jaffe et al., 2004).

A coordinated activity of more than 10 different enzymes is required for porphyrin biosynthesis (Ajioka et al., 2006). One of them, porphobilinogen synthase or δ-aminolevulinic acid dehydratase (δ-ALA-D, E.C. 4.2.1.24, Figure 1), is a metalloprotein involved in the formation of the common porphyrin precursor porphobilinogen (PBG) via a unique asymmetric condensation of 2 molecules of δ-aminolevulinic acid (ALA) and loss of 2 molecules of water (Tang et al., 2006).

Some methodologies have been employed to unravel the molecular mechanism by which the δ-ALA-D from yeast and animals produces PBG, such as enzyme kinetic studies, site-directed mutagenesis, analysis of conformation and position of residues and substrate ALA in different crystal structures (Jaffe, 2004). More recently, Density Functional Theory (DFT) studies have been applied to elucidate its catalysis (Erdtman et al., 2010). Among them, there is a consensus that the catalysis involves covalent Schiff bases linkages between adjacent active site lysine residues (Lys199 and Lys252 in human δ-ALA-D, at A (acetic) and P (propionic)-site, respectively) and an interaction involving Zn(II) ion coordinated to the thiolates of three cysteinyl residues (Cys122, Cys124 and Cys132 in human δ-ALA-D) and

the nitrogen from $–NH_2$ group from one of the ALA substrate (A-site). Lys252 is essential in the catalysis because it is involved in the recognition of N from pyrrole ring (P-site) from PBG (Erdtman et al., 2010).

In the last twenty years, pre-clinical trials have attributed a diversity of pharmacological effects to the organochalcogen molecule diphenyl diselenide [$(PhSe)_2$, Figure 2], including antioxidant, antiulcer, neuroprotective, anti-inflammatory, anti-hyperglycemic, anti-atherosclerotic and anti-hypercholesterolemic properties (de Bem et al, 2009; de Freitas et al., 2010; Nogueira and Rocha, 2011; Hort et al., 2011). On the other hand, $(PhSe)_2$ can also present cytotoxic properties (Posser et al., 2011) and disrupt the activity of thiol-containing proteins (Kade and Rocha, 2010; Puntel et al., 2010; Lugokenski et al., 2011), including δ-ALAD. *In vitro* studies have shown that animal δ-ALAD inhibition occurs via oxidation of their sulfhydryl residues, since dithiothreitol (DTT) recovers $(PhSe)_2$-iinhibited δ-ALAD (Barbosa et al., 1998; Farina et al., 2002; Soares et al., 2005; Fachinetto et al., 2006; Golombieski et al., 2008). Accordingly, the development of novel potentially therapeutic diselenide derivatives with antioxidant properties and low affinity for δ-ALA-D is needed.

A more detailed comprehension on binding affinities of protein-ligand complexes by computational methods has become an important and essential tool in optimizing and finding novel drugs with affinity to a specific target (Mucs and Brice, 2013; Menezes et al., 2003; Menezes et al., 2006 and de Menezes et al., 2008). Additionally, *in silico* toxicological studies concerning the affinity of drugs to enzymes involved in essential physiological processes is also of meaningful importance, since these data could provide the rational development of efficient drugs with less toxic effects (Gleeson et al., 2012).

In line with this, different strategies and methodologies have been exploited, including the use of classical and quantum mechanics calculations (Raha et al., 2007). Although *ab initio* or other quantum calculation methods could provide a more accurate and unbiased interaction energies, it demands a high computational cost when considering the entire protein-ligand complex containing several thousand of atoms due to the very large number of electrons involved. As solution, fragmentation methods have been used on these large complexes to simpler full systems in order to make it more computationally acceptable and, at the same time, maintain the good accuracy of the quantum calculation (Jing and Han, 2010). For this purpose, the Molecular Fractionation with Conjugated Caps (MFCC) method has been pointed as a useful approach to calculate interaction energies for protein-ligand

complexes (Zhang and Zhang, 2003, da Costa et al., 2012, Zanatta et al., 2012). In this method, the entire complex is divided into small subsystems and the calculations are executed individually for each subsystem. The peptide bonds of the protein are fragmented and the bonds are capped with portions of the neighboring amino acid residues of the molecule in order to resemble the local environment (Jing and Han, 2010; Barroso-Neto et al., 2012).

Recently, we modeled the complex formed by the interaction of $(PhSe)_2$ and analogs with human δ-ALA-D by *in silico* molecular docking (Saraiva et al, 2012). According to the proposed model, $(PhSe)_2$ acesses the TIM barrel active site of octameric δ-ALA-D, allowing the close approximation of its selenium atoms to –SH groups of Cys124, Cys122 and Cys132. This is crucial for subsequent oxidation of these cysteyl residues and enzyme inhibition. In addition, an important role of π-π stacking interactions involving Phe208, CH…π interactions with Phe79 and cation-π interactions involving the side chains of Lys199 and Arg209 residues with the aromatic ring of $(PhSe)_2$ could also be observed (Saraiva et al, 2012). However, studies concerning individual energies contribution of each residue in the protein-ligand complex have not yet been carried out. Such type of studies can better explain the interaction of ALA-D with diselenides in details and help the modeling of molecules with low affinity for ALA-D. Here, we aimed to improve our understanding δ-ALA-D inhibition by $(PhSe)_2$ by evaluating the binding energies of each amino acid residue using *in silico* theoretical quantum biochemistry calculations. The results of the present study can provide mechanistic support to the rational design of novel therapeutically viable diselenide drugs with low affinity for δ-ALA-D.

2. General Computational Details

2.1 Preparation of macromolecule and ligand

The crystal structure of human δ-ALA-D obtained from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/) was used as macromolecule, with PDB code 1E51. Hydrogen atoms were previously added to the macromolecule, followed by energy minimization using AM1-BCC (Jakalian et al., 2002) method and partial Gasteiger charges (Gasteiger and Marsili, 1980) implemented in UCSF Chimera 1.5.3 program (Pettersen et al., 2004). The structure of $(PhSe)_2$ was drawn using the program Avogadro 0.9 followed by geometry optimization with the Universal Force Field (UFF) partial charges classical method (Rappé et

al, 1992) and PM3 semi-empirical method (Stewart JJP, 1989ab) implemented in MOPAC2009.

2.2 Molecular docking and geometry optimization of the complex

The complex formed by $(PhSe)_2$ and δ-ALA-D, were previously modeled by *in silico* molecular docking using AutoDock Vina 1.1.1 program (Trott and Olson, 2010; Saraiva et al., 2012). AutoDock Vina is able to operate by pairing an empirically-weighted scoring function enclosing terms for values such as hydrogen bonding, hydrophobic interaction (van der Waals), rotatable bond penalties, and a sophisticated gradient-based local search as a global optimization algorithm. Tridimensional coordinates from crystal structure of human δ-ALA-Dand ligand $(PhSe)_2$ are previously prepared using AutoDock Tools 4.2 (Morris et al., 2009) by adding polar hydrogens and Gasteiger charges. For docking procedure, all rotatable bonds within the ligands were allowed to rotate freely while the macromolecule was kept rigid. The grid box was centered at coordinates x = 30.81, y = 23.662 and z = 9.581 and dimensions of 68 Å × 57 Å × 66 Å points. The spacing consisted of 1 Å and the exhaustiveness was set at 200. All other docking parameters were used as defaults. The conformation generated with the lowest binding free energy was accepted as the most probable model of interaction. Briefly, a redocking using the co-crystalized ligand porphobilinogen (PBG) was also performed, using the same adopted docking conditions. The RMSD from the best pose of ligand PBG proposed by AutoDock Vina program and the real conformation of PBG in the crystal was of 1.074 Å (Saraiva et al., 2012), suggesting that AutoDock Vina 1.1.1 program can propose a reliable model of interaction (RMSD < 2.0 Å). After getting the complex formed by $(PhSe)_2$ and δ-ALA-D by molecular docking, all missing hydrogen atoms were added and their positions were classically optimized, maintaining them flexible while the other atoms were fixed. The geometry optimization procedure was performed using the Forcite code, with the UFF partial charges (due to the presence of selenium atoms). The convergence tolerances were set to 2.0×10^{-5} kcal·mol^{-1} for total energy variation, 0.001 kcal·mol^{-1}·Å^{-1} for maximum force per atom, and 1.0×10^{-5} Å for maximum atomic displacement.

2.3. DFT calculations

The calculations at DFT level were carried out using the DMol^3 code (Delley B, 2000), using: i) the Local Density Approximation (LDA) for the exchange-correlation functional with PWC parameterization (Delley B, 1990), and 2) the Generalized Gradient

Approximation (GGA) (Perdew et al., 1992) parameterized by Perdew Wang 91 functional (PW91) (Perdew et al., 1996), both with Ortmann, Bechstedt, and Schmidt (OBS) damped atom-pairwise dispersion corrections for DFT (Ortmann et al., 2006). In order to expand the Kohn-Sham orbital for all electrons, a double numerical plus polarization (DNP) numerical basis set was adopted. The DNP basis set is comparable to 6-31G** Gaussian basis sets, but with much more accuracy (Inada and Orita, 2008). The orbital cutoff was set to 3.7 Å and the smearing was set of 0.005 Ha. The total energy variation, which specifies the self-consistent field (SCF) convergence threshold, was set to 10^{-6} Ha, ensuring a well converged electronic structure for the system.

It is well established in the literature that pure DFT methods can not accurately describe systems where noncovalent bonding is pertinent (Zhao et al., 2005, Cooper et al, 2008 Silvestrelli et al, 2009). In addition, it is known that LDA functional may overestimate relative energy values when compared to experimental energies (mainly when ions and charged moieties are considered) and is not adequate to characterize hydrogen bonds (Zanatta et al., 2012). On the other hand, theoretical studies have reported that LDA can be used to treat systems where noncovalent bonding is essential to the interaction and system stabilization (e. g. graphite, hydrated guanine crystals and aromatic binding of ligands to an enzyme) (Ortmann et al., 2005, 2006, 2008; Kee et al., 2009). Furthermore, LDA can estimate relative energies correlated with those obtained by experimental data, thus giving an improved scenario of the general behavior tendencies of interaction (da Costa et al., 2012). Moreover, studies concerning aromatic interaction of ligands binding enzymes have shown that LDA functional has a better agreement with the more sophisticated MP2 method when compared to hybrid functionals (da Costa et al., 2012; Zanatta et al., 2012). Regarding calculations based on GGA with PW91 functional, studies have pointed that it yield a good prediction of Van der Waals interactions in organic and hydrogen bonded systems (Tsuzuki and Lüthi, 2001; Alfonso et al., 2004). PW91 can also be highly useful for computational studies of neutral and cationic conformers of aromatic molecules with flexible side chains (Patey and Dessent, 2002). Together, these evidences allowed us to set both the LDA/PWC and GGA/PW91 functionals as a way to obtaining a better understanding what δ-ALA-D amino acids could exert an important role in the interaction with the inhibitor $(PhSe)_2$.

2.4. Molecular Fractionation with Conjugated Caps (MFCC)

In order to estimate relative energies of interaction between $(PhSe)_2$ and each neighboring amino acid residue belonging or close to the active center site of δ-ALA-D, quantum mechanics studies using the Molecular Fractionation with Conjugated Caps (MFCC) method (Zhang and Zhang, 2003; Antony and Grimme, 2012; da Costa et al., 2012) were performed. Here, the tridimensional structure of the protein-ligand complex is decomposed into individual amino acid-based fragments that are treated with proper molecular caps.

According to MFCC method, the interaction (binding) energy $E(L\text{-}R_i)$ (expressed in kcal mol^{-1}) between a ligand L and an amino acid residue R_i from the enzyme is given by the equation (1):

$$E(L\text{-}R_i) = E(L\text{-}C_{i-1}R_iC_{i+1}) - E(C_{i-1}R_iC_{i+1}) - E(L\text{-}C_{i-1}C_{i+1}) + E(C_{i-1}C_{i+1}) \quad (1)$$

where C_{i-1} and C_{i+1} are the caps obtained from neighbor residues to R_i (R_{i-1} and R_{i+1}, respectively); $E(L\text{-}C_{i-1}R_iC_{i+1})$ is the total energy of the subsystem consisted of ligand L, residue R_i and caps C_{i-1} and C_{i+1}; $E(C_{i-1}R_iC_{i+1})$ is the total energy of the subsystem consisted of residue R_i and caps (Fig. 3).

However, the high proximity of the ligand $(PhSe)_2$ to the thiolates from residues Cys122, Cys124 and Cys132 bound to Zn(II) ion may influence the charge density of $(PhSe)_2$. Thus, we decided to include in the calculations a special cap formed by Cys122, Cys124 and Cys132 bound to Zn(II) (denominated here as $C_{Cys\text{-}Zn}$) beside neighbor caps C_{i-1} and C_{i+1} in each individual amino acid-based fragment, for a more realistic prediction of the interaction energies between each individual residue and $(PhSe)_2$ (Figure 4, equation 2).

$$E(L\text{-}R_i) = E(L\text{-}C_{i-1}R_iC_{i+1}\text{-}C_{Cys\text{-}Zn}) - E(C_{i-1}R_iC_{i+1}\text{-}C_{Cys\text{-}Zn}) - E(L\text{-}C_{i-1}C_{i+1}\text{-}C_{Cys\text{-}Zn}) + E(C_{i-1}C_{i+1}\text{-}C_{Cys\text{-}Zn}) \quad (2)$$

The total interaction energy of ligand $(PhSe)_2$ binding δ-ALA-D enzyme **[E(L-δ-ALA-D)]** is approximated by the summation of all individual energies calculated from N amino acid residues of enzyme and ligand $[E(L\text{-}R_i)]$ and is given by the equation (3):

$$E(L\text{-}\delta\text{-}ALA\text{-}D) = \sum_{i=1}^{N} E(L\text{-}R_i) \quad (3)$$

The binding pocket was defined as an imaginary sphere surrounding δ-ALA-D residues close to the ligand $(PhSe)_2$ (from the TIM barrel active site) and, consequently, the center of the binding pocket was determined as the ligand centroid (see Figure 2 and Table 1). When considering the order of energy calculations from each amino acid, the distances from the ligand centroid to each corresponding amino acid residue in the complex proposed by molecular docking were taken into account, at distances ranging from 2.5 to 12.0 Å (with intervals of 0.5 Å), and the calculations were stopped when an energy convergence was achieved, i.e., when the variation of the total binding energy was smaller than 10% after a radius size increased by 2.0 Å.

3. Results and discussion

In our study, the MFCC computational method provided an easy means to study the $(PhSe)_2$/δ-ALA-D complex by explicitly calculating the interaction energy between individual residues and the ligand at QM levels. These individual residue–ligand interaction energies could provide detailed quantitative information about specific residue interactions with the $(PhSe)_2$ that can be informative for understanding the molecular nature of enzyme inhibition. Both LDA/PWC and GGA/PW91 functionals implemented in quantum MFCC method were skilled to present relative energies of residues close to $(PhSe)_2$ at a radius from 2.5 to 12.0 Å of ligand centroid (Figure 6). Despite differences in the relative values of energies calculated by LDA (E_{LDA}) and GGA (E_{GGA}) is observed, however, a good correlation between them is noted ($R^2 = 0.817$, Figure 9). In figures 6 and 8, the variation of the interaction energies as a function of the δ-ALA-D binding pocket radius E(*r*) is shown. It is possible to note that the energy convergence is achieved at a 8.5 Å distance (Figure 6, 7), where the amino acid residues with the most important attractive or repulsive interactions to $(PhSe)_2$ are found. Calculated energies from each residue are given in Table 2. A positive correlation between the relative energy values obtained After convergence, the total relative binding energy E(L-δ-ALA-D) was estimated to be –187.03 kcal·mol^{-1} for LDA/PWC and –65.53 kcal·mol^{-1} for GGA/PW91.

The energies from the most important amino acid residues were plotted into a detailed graph where we denominated here “Binding site, Interaction energy and Residues Domain” (BIRD) panel (Figure 8) (da Costa et al., 2010). The BIRD panel is consisted of: (i) horizontal bars showing the binding energy (in kcal·mol^{-1}) of $(PhSe)_2$ to the most important residues (left-aligned), from which one can assign quantitatively the role of each residue in the binding

site (as attractive or repulsive forces); (ii) the distance from ligand centroid (right-aligned); and (iii) the region of the ligand (e. g., "iii (C11)H" shown in Figure 2) which is closer to the respective residue of the binding site.

According to the BIRD panel (Figure 8), the residues Lys199 (E_{LDA} = –23.752 kcal·mol^{-1}; E_{GGA} = –19.754 kcal·mol^{-1}); Phe208 (E_{LDA} = –18.340 kcal·mol^{-1}; E_{GGA} = –11.107 kcal·mol^{-1}), Lys252 (E_{LDA} = –16.821 kcal·mol^{-1}; E_{GGA} = –14.652 kcal·mol^{-1}) Phe79 (E_{LDA} = –11.742 kcal·mol^{-1}; E_{GGA}= –6.924 kcal·mol^{-1}), Cys132 (E_{LDA} = –18.821 kcal·mol^{-1}; E_{GGA} = –22.33 kcal·mol^{-1}) and Cys124 (E_{LDA} = –11.746 kcal·mol^{-1}; E_{GGA} = –6.755 kcal·mol^{-1}) can exert the strongest attractive energy on $(PhSe)_2$. The residues Asp120, Arg209, Ala212, Arg221, Tyr205, Tyr126, Gln225 and Val81 also exert attractive energies (see energy values in Figures 6, 7-B, 8-B and Table 3) important in the complex stabilization.

The energy calculations for the positive charged residues Lys199, Lys252, Arg209 and Arg221 (Figures 10-C and 11-B) were found as exerting attractive energy (Figure 8 and Table 3). According to the ligand and residues orientation and conformation, it is expected that the phenyl moiety of $(PhSe)_2$ could be strongly attracted by positive charged residues Lys199 via cation-π stacking interactions (Figures 7-C and 8-C).

The orientation of the phenyl moiety of Phe208 close to the aromatic region iii (C7-C12)H of $(PhSe)_2$ (Figures 7-D and 8-D) exhibits a π-π stacking noncovalent interaction (Saraiva et al., 2012). In addition, the strong attraction energy of the thiolates (nucleophile species) of Cys124, Cys122 and Cys132 bound to Zn(II) (Figures 10-A and 11-A) involves the selenium atoms of $(PhSe)_2$ (which here is the electrophile species). This interaction is crucial for a redox reaction, leading to the reduction of $(PhSe)_2$ into 2 molecules of phenylselenol (PhSeH) and subsequent catalytic cystenyl oxidation and enzyme inhibition (Farina et al., 2002; Saraiva et al., 2012).

Taking into account the interaction of 2 molecules of the substrate ALA into δ-ALA-D, we can divide its active center site into two regions: A-site (that is related to the acetic moiety from PBG) and P-site (that is related to the propionic moiety from PBG). The residues Lys252 (A-site) and Lys199 (P-site) are involved in Schiff-base linkages to ALA substrates and previous experimental mutagenesis studies have suggested that Lys252 is essential for catalysis (Jaffe, 2004; Erdtman et al., 2010). In addition, several polar groups hydrogen bond to the carboxylates of the P- (Tyr126) is able to bind ALA moieties and other amino acid

residues (Asp120 and Tyr205) constitute a polar pocket around, or hydrogen bond to the terminal amino groups of the ALA substrates (Erdtman et al., 2010). Thus, the strong interaction between $(PhSe)_2$ and these catalytic residues is a indicative of an efficient inhibition.

Here, the use of theoretical biochemistry calculation using the MFCC method pointed out the interactions between $(PhSe)_2$ and δ-ALA-D and the important residues involved in the stabilization of the complex prior to the cysteine oxidation. Our analysis indicated that the toxicity of $(PhSe)_2$ can be associated with the presence of aromatic moieties, which increases $(PhSe)_2$ affinity with aromatic and positively charged residues in the target protein. Additionally, these interactions of phenyl moieties approximate the electrophile selenium atoms close to cysteine catalytic residues. This subsequently leads to oxidation of cysteyl residues in the active center of δ-ALA-D. In conclusion, these data indicate that introduction of bulkier substituent groups to aromatic ring in $(PhSe)_2$ than that previously tested (Saraiva et al. 2012; Rocha et al., 2012) could decrease the inhibitory potency of $(PhSe)_2$ and analogs towards δ-ALA-D. Thus, the results presented here provided some important theoretical information that can help in the rational drug design of novel diselenides with less toxicity, when δ-ALA-D is considered the molecular endpoint of toxicity.

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Conflict of interest

The authors declare no conflict of interest.

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FIGURES

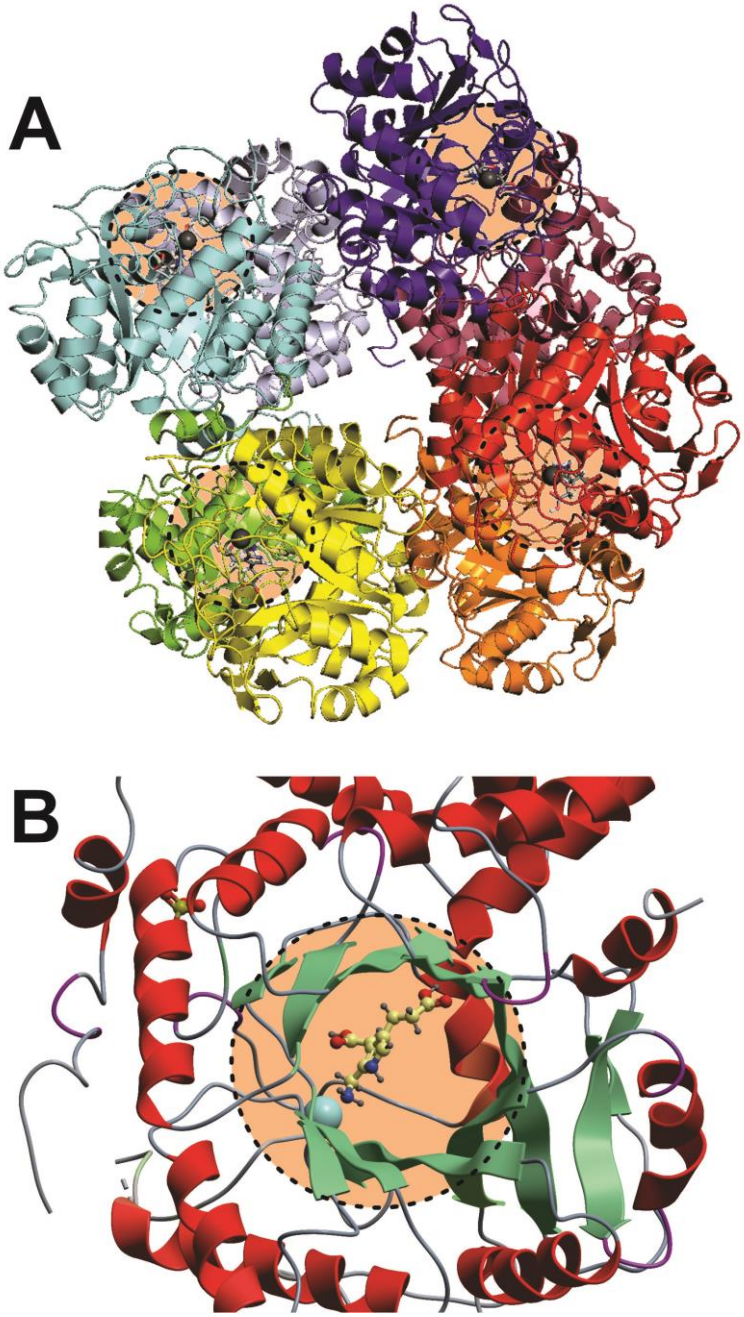

Figure 1 – Human δ-ALA-D (PDB code 1E51). (A): Octamer structure showing four active sites (each monomer is indicated with a different color); (B): Monomer structure showing the TIM barrel binding site of δ-ALA-D complexed with the co-crystalized product porphobilinogen (PBG). The residues belonging to the binding site are inside the orange dashed spheres of radius *r* surrounding the PBG molecule.

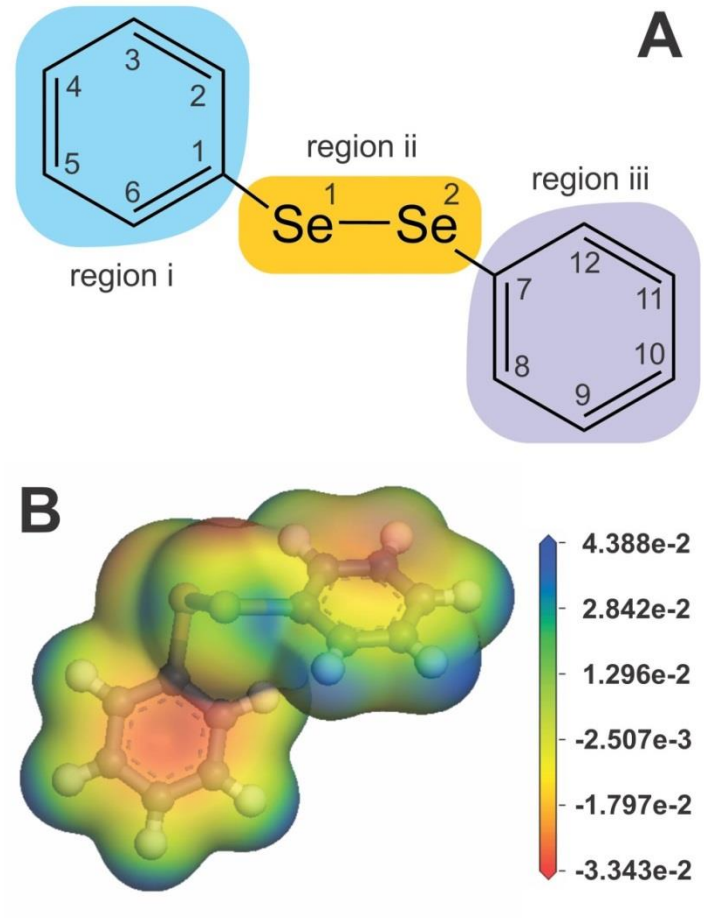

Figure 2 – Structure of diphenyl diselenide [$(PhSe)_2$]. (A) 2D chemical structure with atoms labeled by numbers. Regions i and iii pointed out aromatic moieties. Region ii showed selenium atoms in the molecule. (B) Electron density of $(PhSe)_2$ (with 3D conformation oriented into the active center site of human δ-ALA-D) projected onto an electrostatic potential isosurface (isovalue= 0.017) showing negatively charged regions (tended to red) and positively charged regions (tended to blue) calculated by DFT (at LDA/PWC functional level using $DMol^3$ code).

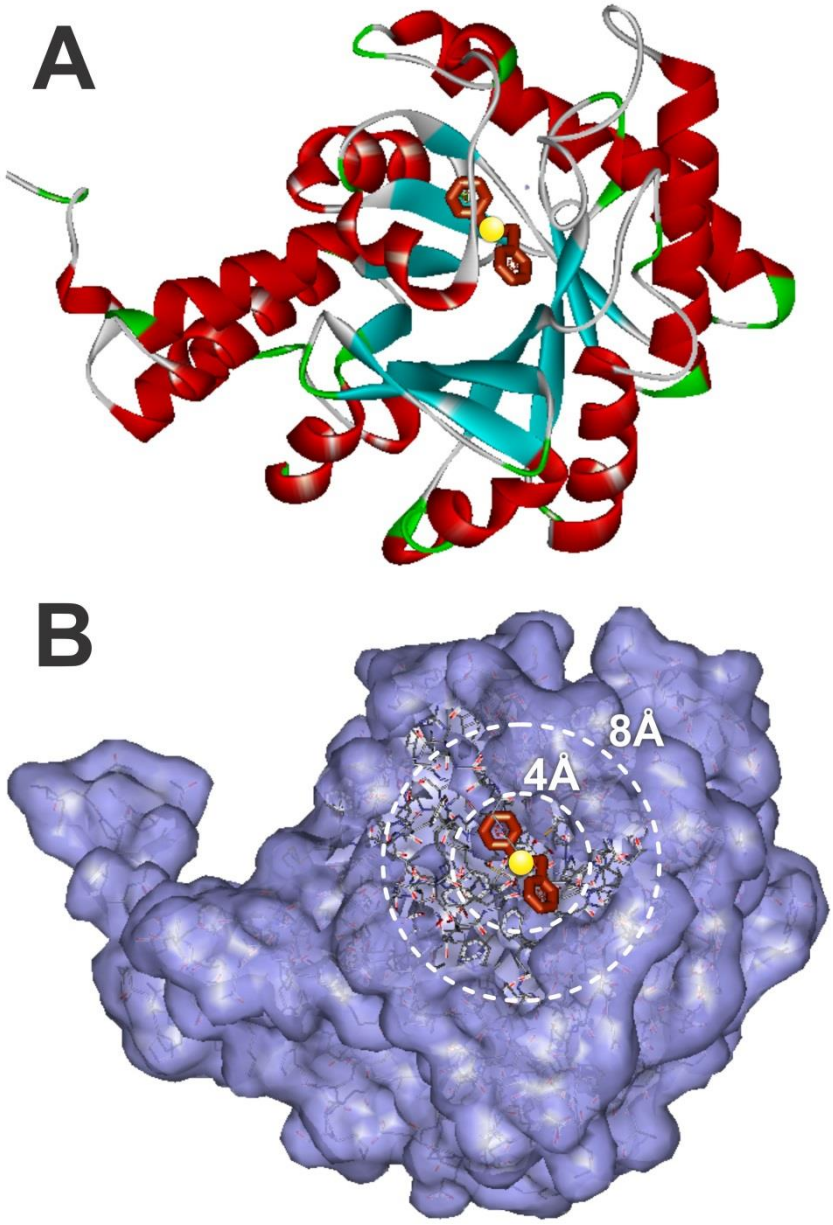

Figure 3 – Diphenyl diselenide [$(PhSe)_2$] in complex with the monomer δ-ALA-D. Yellow centered ball denotes the ligand centroid. (A) Secondary structure representation showing $(PhSe)_2$ bound δ-ALA-D TIM barrel active site. (B) Surface representation. Dashed white circumferences denote different radii (4 Å and 8 Å) from ligand centroid.

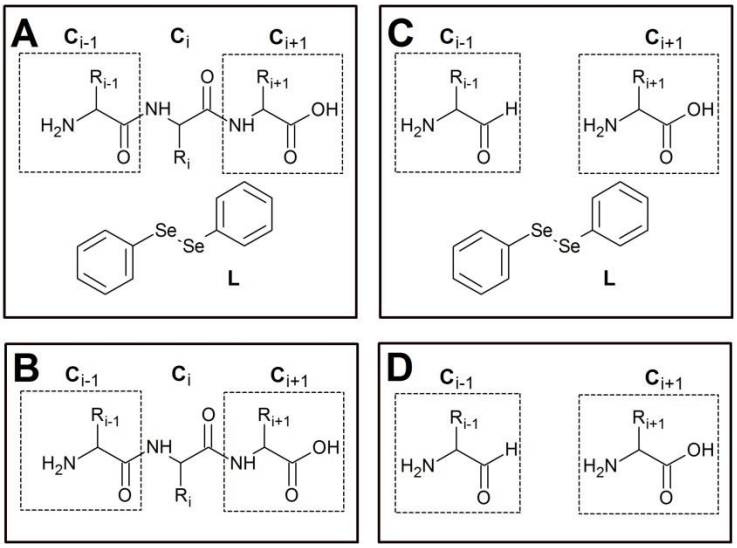

Figure 4 – MFCC scheme showing fragmented systems employed in general calculations.

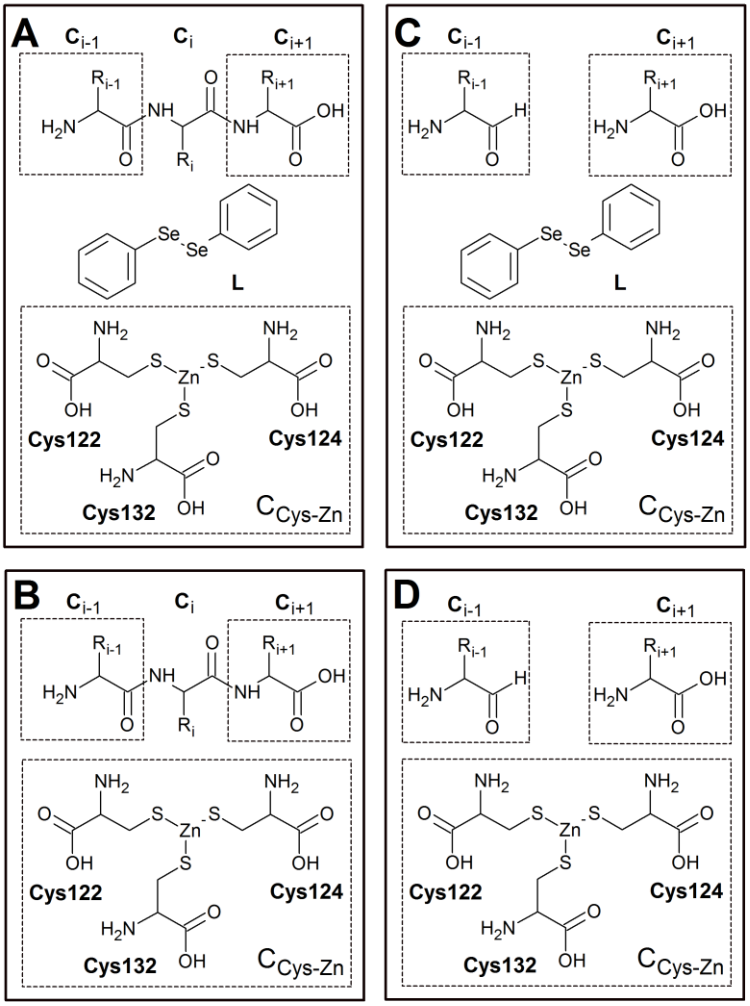

Figure 5 –MFCC scheme showing fragmented systems with addition of subsystem C_{Cys-Zn}.

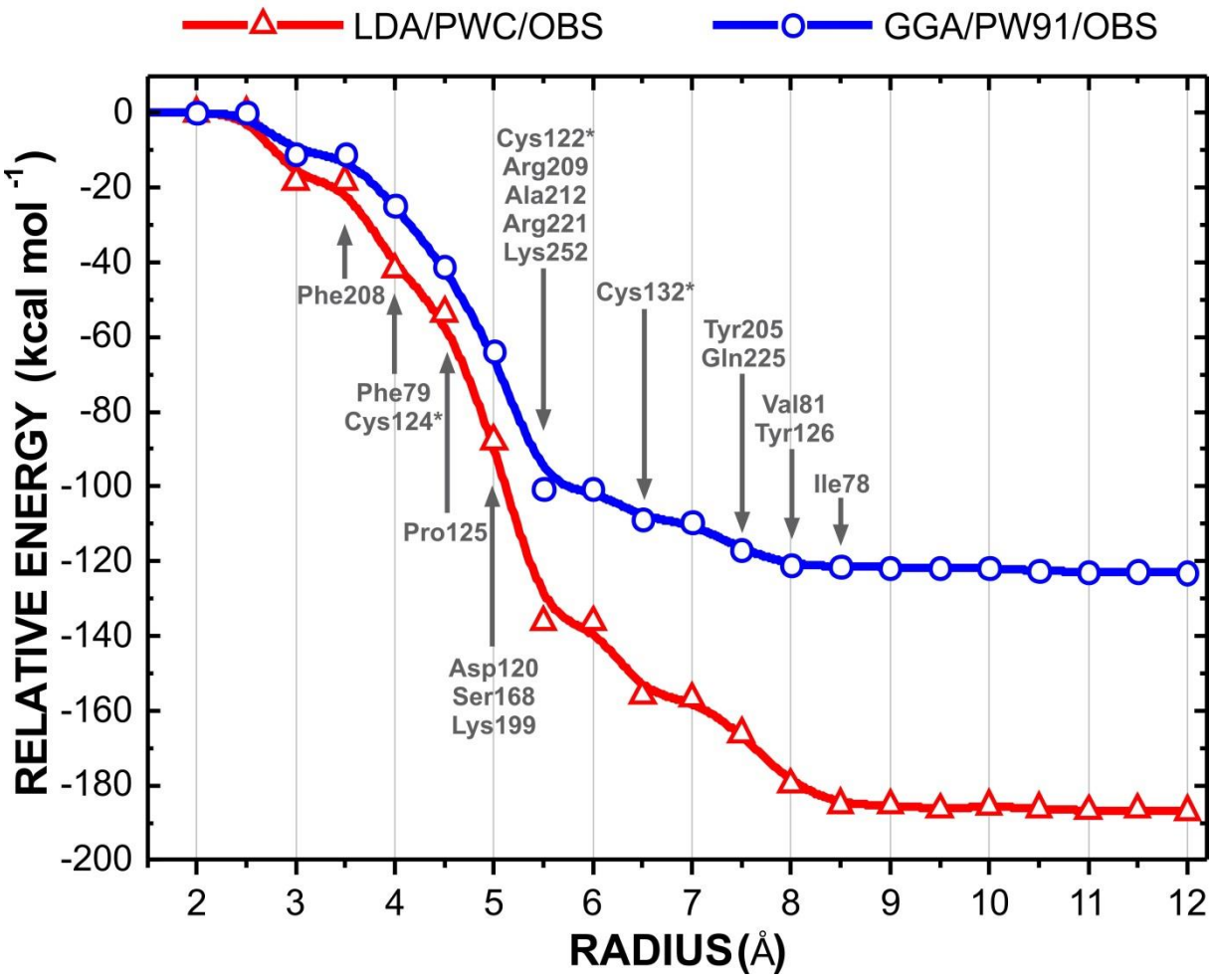

Figure 6 – Variation of the DFT (LDA/PWC and GGA/PWC) relative interaction energies as a function of the binding pocket radius. Arrows indicate the first appearance of residues in a given radius. Thiolates instead thiols were considered for Cys124*, Cys122* and Cys132* side chains during the energies calculation.

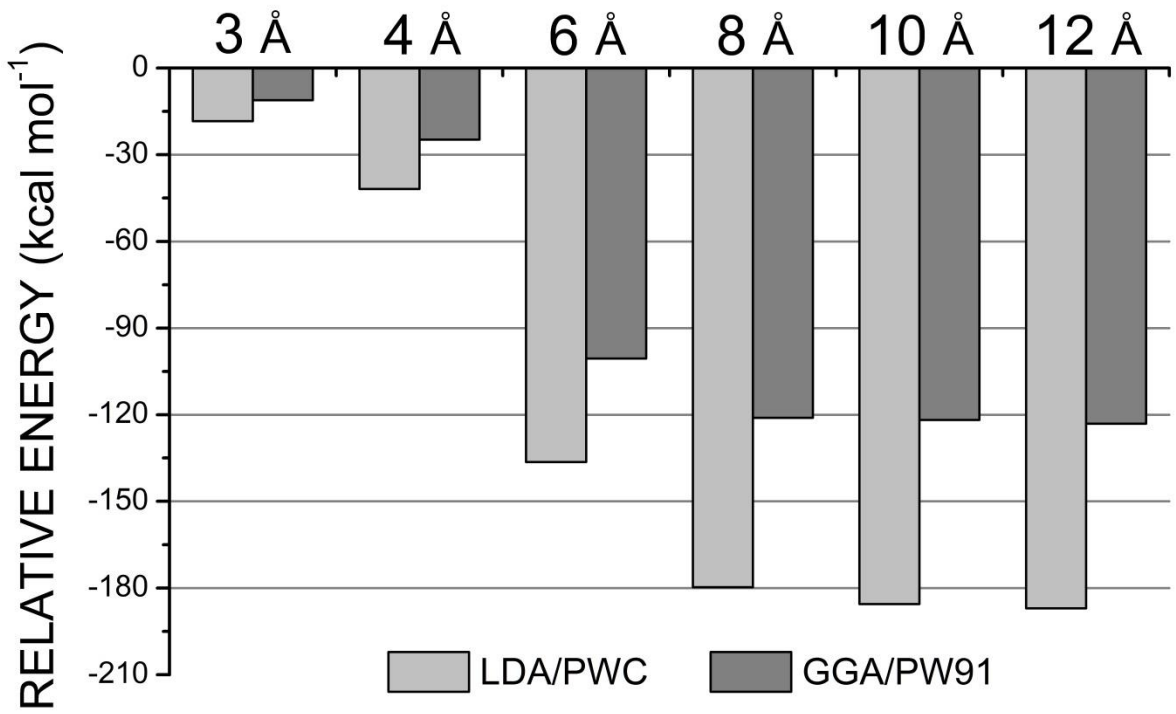

Figure 7 – Total DFT interaction energies of $(PhSe)_2$ for binding δ-ALA-D pocket radii of 3 Å, 4 Å, 6 Å, 8 Å, 10 Å and 12 Å.

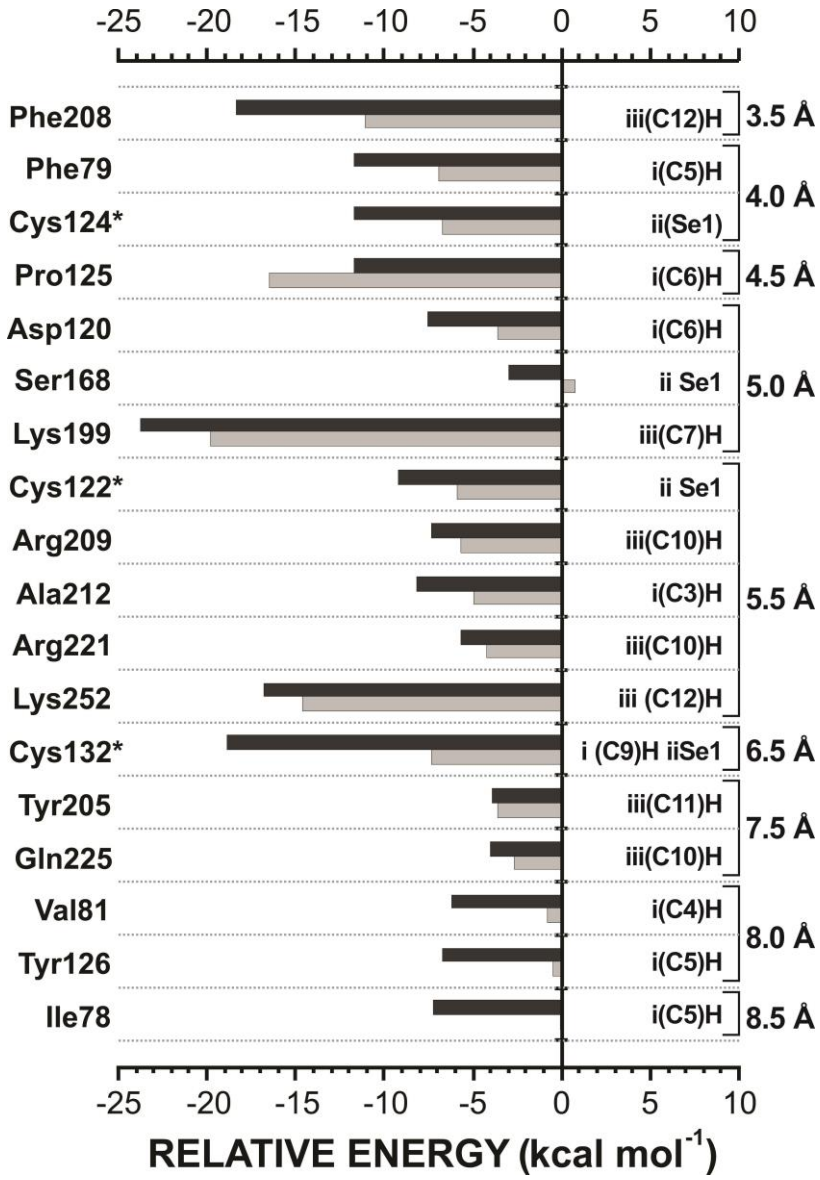

Figure 8 – BIRD graphic panel showing the interaction energy for the amino acid residues with relative interaction energies < –5.0 kcal·mol^{-1} (attractive energies) or > 5.0 kcal·mol^{-1} (repulsive energies). Black bars indicate LDA/PWC relative energies and gray bars, GGA/PW91. Here, the most important residues for complex interaction are shown, according to DFT calculations. The binding pocket radii (taking into the consideration the ligand centroid) for each amino acid is right-aligned.

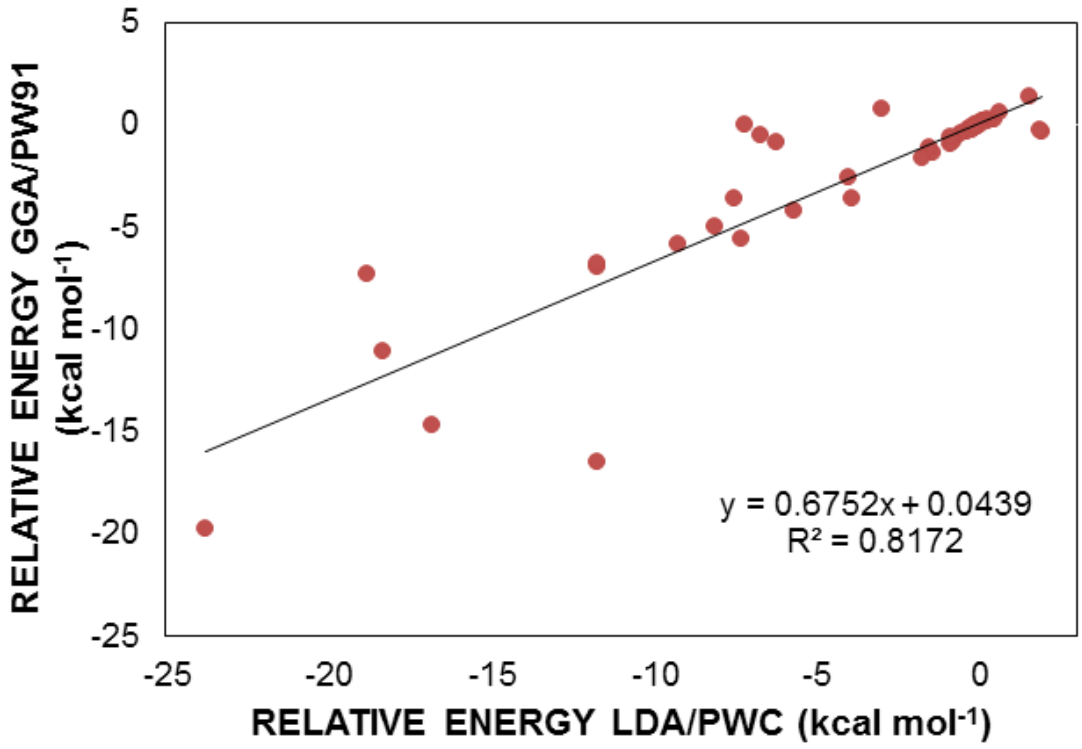

Figure 9 – Linear correlation between GGA/PW91 and LDA/PWC relative energies of obtained by MFCC method.

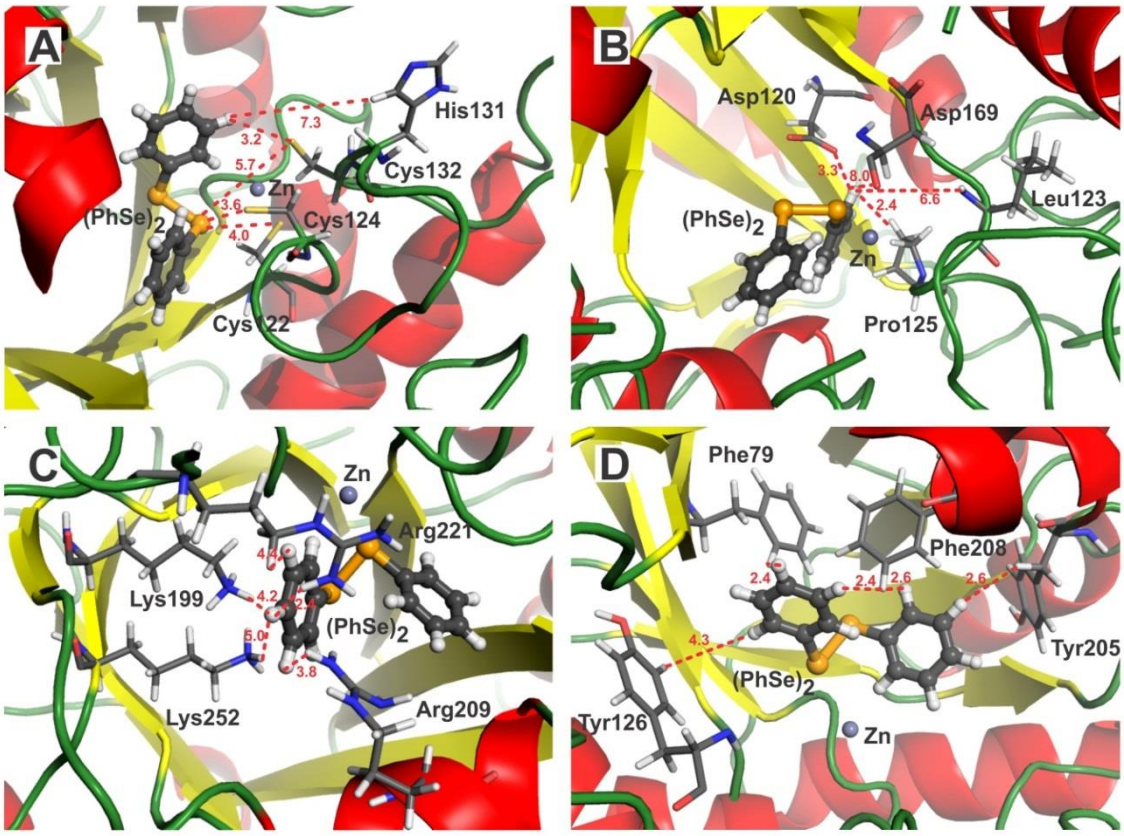

Figure 10 – Interaction of $(PhSe)_2$ and the most attractive and repulsive residues from δ-ALA-D binding site. Red dashed lines and labeled number denote the shorter distance (in angstroms) between each amino acid residue and ligand $(PhSe)_2$. Residues are represented as stick and $(PhSe)_2$ as ball-and-stick. Atoms are colored as follows: carbon dark grey, nitrogen blue, oxygen red, hydrogen white, sulfur yellow and selenium dark yellow. Zinc ion is represented as a light grey sphere.

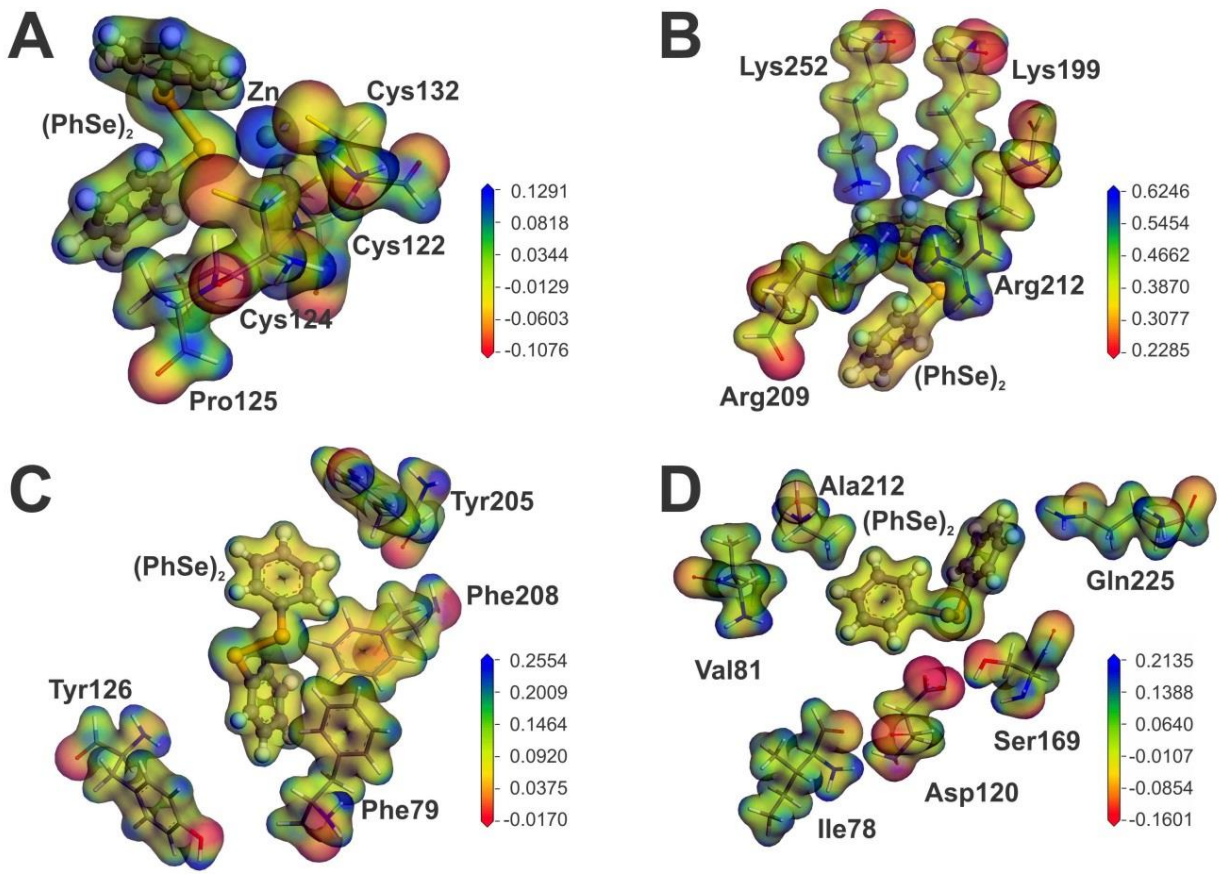

Figure 11 – Electron map densities projected onto relative electrostatic potential isosurfaces (value = 0.2) around $(PhSe)_2$, and the most attractive residues from δ-ALA-D binding site, calculated at DFT/LDA/PWC level. (A) cysteinyl residues Cys122, Cys124 and Cys132 complexed to Zn(II); (B) positive-charged residues Lys199, Lys252 (involved in cation-π interactions), Arg209 and Arg221; (C) aromatic residues Phe208, Phe79, Tyr205 and Tyr126 (most of them involved in π-π stacking interactions); (D) . Negatively charged regions (tended to red) and positively charged regions (tended to blue) are shown.

TABLES

Table 1 – Residues found in δ-ALA-D active center site at radii ranging from 2.5 to 12.0 Å, taking into account the spatial position of $(PhSe)_2$ ligand centroid. Main residues for complex interaction are highlighted in boldface font. In blue are shown positive residues, in red negative residues and in green Zn(II) ion is denoted.

Radius (Å)	**Residues (R_i)**							
2.5	**Phe208**							
3.0	—							
3.5	—							
4.0	**Phe79**	**Cys124**						
4.5	**Pro125**	**Zn(II)**						
5.0	Asp120	Ser168	**Lys199**					
5.5	Cys122	Arg209	Ala212	Ser214	Arg221	**Lys252**		
6.0	—							
6.5	Phe36	Cys132						
7.0	Tyr196							
7.5	Gly80	Leu123	Tyr205	Gln225	Tyr276			
8.0	Leu77	Val81	Val121	Tyr126	Gly130	Asp169	Ala211	
8.5	Ile78	Tyr224	Tyr318					
9.0	His131	Ala166	Pro167	Lys213	Ser215	Pro216		
9.5	Pro34	Pro82	Thr127	Val278	Ser279			
10.0	Cys119	Leu150	Met170	Met250				
10.5	Ile35	Lys87	Gly133	Tyr157	Arg174	Pro207	Asp210	Ile316
11.0	Val37	Ile47	Leu50	Ala94	Ala101	Gly206		
11.5	Arg55	His129	Val153	Met171	Phe200	Ala201	Phe204	
12.0	Pro100	Ser202	Ala217	Pro253	Gly280			

Table 2 – Individual amino acid residue contribution as a function of the radius size. Energies calculated by LDA/PWC (E_{LDA}) and by GGA/PW91 (E_{GGA}) are expressed in kcal·mol^{-1} and radii (r) from ligand centroid are given in Å.

Residue (R_i)	**E_{LDA}**	**E_{GGA}**	**r (Å)**	**Residue (R_i)**	**E_{LDA}**	**E_{GGA}**	**r (Å)**
Phe208	-18.340	-11.107	3.5	Pro216	-0.125	-0.105	9.0
Phe79	-11.742	-6.924	4.0	Pro34	-0.242	-0.212	9.5
Cys124	-11.746	-6.755	4.0	Pro82	-0.151	-0.147	9.5
Pro125	-11.734	-16.464	4.5	Thr127	-0.058	-0.057	9.5
Asp120	-7.501	-3.592	5.0	Val278	-0.345	-0.293	9.5
Ser168	-2.992	0.796	5.0	Ser279	-0.260	-0.227	9.5
Lys199	-23.752	-19.754	5.0	Cys119	0.146	0.152	10.0
Cys122	-9.251	-5.855	5.5	Leu150	0.036	0.053	10.0
Arg209	-7.324	-5.632	5.5	Met170	0.658	0.593	10.0
Ala212	-8.127	-4.961	5.5	Met250	0.011	-0.003	10.0
Ser214	-1.419	-1.374	5.5	Ile35	0.027	0.026	10.5
Arg221	-5.652	-4.256	5.5	Lys87	1.559	1.370	10.5
Lys252	-16.821	-14.652	5.5	Gly133	-0.064	-0.068	10.5
Phe36	-0.739	-0.818	6.5	Tyr157	-0.117	-0.114	10.5
Cys132	-18.826	-7.341	6.5	Arg174	-0.795	-0.870	10.5
Tyr196	-0.867	-0.923	7.0	Pro207	-0.388	-0.317	10.5
Gly80	-1.544	-1.103	7.5	Asp210	-0.863	-0.630	10.5
Leu123	0.254	0.259	7.5	Ile316	-0.084	-0.072	10.5
Tyr205	-3.895	-3.584	7.5	Val37	-0.205	-0.176	11.0
Gln225	-3.986	-2.629	7.5	Ile47	-0.087	-0.053	11.0
Tyr276	-0.223	-0.237	7.5	Leu50	-0.105	-0.068	11.0
Leu77	-0.358	-0.344	8.0	Ala94	0.065	0.061	11.0
Val81	-6.214	-0.840	8.0	Ala101	-0.066	-0.051	11.0
Val121	-0.150	-0.188	8.0	Gly206	-0.186	-0.153	11.0
Tyr126	-6.699	-0.493	8.0	Arg55	0.252	0.124	11.5
Gly130	-0.270	-0.211	8.0	His129	-0.169	-0.130	11.5
Asp169	-1.729	-1.648	8.0	Val153	-0.051	-0.054	11.5
Ala211	1.915	-0.377	8.0	Met171	0.165	0.153	11.5
Ile78	-7.213	-0.027	8.5	Phe200	0.127	0.159	11.5
Tyr224	1.867	-0.271	8.5	Ala201	-0.066	-0.045	11.5
Tyr318	-0.112	-0.081	8.5	Phe204	0.028	0.035	11.5
His131	-0.100	-0.085	9.0	Pro100	-0.098	-0.073	12.0
Ala166	0.185	0.162	9.0	Ser202	-0.247	-0.238	12.0
Pro167	-0.536	-0.434	9.0	Ala217	-0.004	0.000	12.0
Lys213	0.496	0.213	9.0	Pro253	-0.018	0.004	12.0
Ser215	-0.049	-0.066	9.0	Gly280	-0.115	-0.096	12.0

Table 3 – Shorter distance between $(PhSe)_2$ and each amino acid residue at the binding site (until 12 Å of ligand centroid) after inserting and optimizing hydrogen atomic coordinates (see the highlighted regions of $(PhSe)_2$ in the figure 2). Main residues for complex interaction are denoted in boldface.

Residue	Distance (Å)	$(PhSe)_2$	Residue	Distance (Å)	$(PhSe)_2$
Phe208	**2.95**	**iii (C12)H**	Pro216	6.10	iii (CH9)H
Phe79	**2.21**	**i(C5)H**	Pro34	8.18	ii Se2
Cys124	**3.43**	**ii Se1**	Pro82	5.17	i (C4)H
Pro125	**2.16**	**i(C6)H**	Thr127	8.29	i (C1)H
Asp120	2.07	i (C6)H	Val278	6.13	iii (C12)H
Ser168	3.53	ii Se1	Ser279	6.16	iii (C11)H
Lys199	**2.58**	**iii (C7)H**	Cys119	6.76	i (C6)H
Cys122	**3.36**	**ii Se1**	Leu150	8.00	ii Se1
Arg209	2.94	iii(C10)H	Met170	6.49	iii (C9)H
Ala212	1.62	i (C3)H	Met250	6.18	iii (C9)H
Ser214	4.46	i(C3)H	Ile35	6.92	i (C5)H
Arg221	7.07	iii(C10)H	Lys87	7.70	i (C3)H
Lys252	**1.96**	**iii (C12)H**	Gly133	8.18	iii (CH9)H
Phe36	3.82	i(C4)H	Tyr157	5.92	i (C5)H
Cys132	**3.08**	**i(C9)H**	Arg174	7.98	iii (CH8)H
Tyr196	4.81	ii Se2	Pro207	8.27	iii (CH11)H
Gly80	1.89	i (C5)H	Asp210	8.32	i (C3)H
Leu123	5.64	ii Se1	Ile316	9.03	ii Se2
Tyr205	2.96	iii (C11)H	Val37	5.42	i (C4)H
Gln225	2.15	iii (C10)H	Ile47	8.02	i (C3)H
Tyr276	5.45	iii (C12)H	Leu50	8.96	iii (C12)H
Leu77	6.72	i (C6)H	Ala94	8.57	i (C5)H
Val81	3.23	i (C4)H	Ala101	5.29	i (C5)H
Val121	4.61	i (C6)H	Gly206	6.92	iii (C11)H
Tyr126	3.66	i (C5)H	Arg55	7.05	i (C4)H
Gly130	7.79	i (C2)H	His129	9.53	iii (C9)H
Asp169	4.99	i (C8)H	Val153	7.47	i (C6)H
Ala211	5.38	i (C3)H	Met171	8.49	iii (C8)H
Ile78	4.39	i (C5)H	Phe200	6.56	iii (C10)H
Tyr224	4.13	iii (C9)H	Ala201	6.11	iii (C10)H
Tyr318	6.37	iii (C12)H	Phe204	6.87	iii (C11)H
His131	6.10	iii (C9)H	Pro100	6.91	i (C4)H
Ala166	6.46	i (C6)H	Ser202	6.65	i (C11)H
Pro167	7.41	iii (C7)H	Ala217	9.97	iii (C9)H
Lys213	5.58	i (C3)H	Pro253	7.15	iii (C11)H
Ser215	7.07	iii (C10)H	Gly280	7.91	iii (C11)H

4 DISCUSSÃO

Por seu envolvimento direto na síntese dos compostos tetrapirrólicos, a expressão e atividade da enzima δ-ALA-D é essencial para manter a homeostase fisiológica nos seres vivos. Consequentemente, a ação de agentes tóxicos exógenos, espécies reativas e outros metabólitos endógenos que inibam sua atividade podem trazer efeitos negativos significativos no metabolismo celular (HEINEMANN et al., 2008). Sendo assim, a compreensão dos mecanismos moleculares envolvendo a interação de reagentes tóxicos na atividade da δ-ALA-D é de extrema importância para as ciências da saúde, agropecuárias e ambientais.

Num primeiro momento, foi realizada uma revisão de literatura visando conhecer informações já existentes quanto à influência de metais, compostos orgânicos e outras situações pró-oxidantes na atividade da δ-ALA-D, demonstrando que a avaliação de sua atividade em diferentes materiais biológicos é uma excelente estratégia para evidenciar a intoxicação por exposição a agentes exógenos e investigar condições fisiopatológicas variadas (Artigo 1). Em condições catalíticas normais, os estudos cristalográficos revelam que a enzima δ-ALA-D de animais (humanos, camundongos) e leveduras (*Saccharomyces cerevisiae*) apresenta no seu sítio catalítico três resíduos de cisteína (Cys122, Cys124 e Cys132 na enzima humana; Cys133, Cys135 e Cys143 na enzima da levedura) cujos tiolatos encontram-se coordenados a um íon Zn(II) (Figura 2-B e Figura 7-A, Artigo 1). É conhecido que o Zn(II) forma complexos mais estáveis com doadores macios (moles), de forma que não é surpresa que ele seja encontrado em peptídeos e proteínas ligados a resíduos de histidina (enzima conversora de angiotensina) ou cisteína (metalotioneínas, anidrase carbônica, álcool desidrogenase) ou em ambos os resíduos (fatores de transcrição contendo “dedos de zinco”) realizando até quatro ligações coordenadas (BERG, 1990; SANGHANI et al., 2002; NATESH et al., 2003; BELL & VALEE, 2009). Além disso, o Zn(II) é um excelente ácido de Lewis, sendo bem adequado para catalisar reações ácido-base devido à sua capacidade de formar ligações fortes com grupos doadores de resíduos de aminoácidos (N e S) e ligantes exógenos (H_2O, por exemplo), e permitir reações de oxidorredução nas interconvenções cisteína-cistina, apesar da sua própria falta de química de oxidorredução (o que é uma vantagem para a catálise em sistemas biológicos) (FRAUSTO DA SILVA & WILLIAMS, 2001; BELL & VALEE, 2007).

No entanto, é evidenciado que outros metais e compostos metálicos [Pb(II), Hg(II), Cd(II), CH3Hg(II), Ga(III), In(III), Sn(II), Bi(III)], e não-metais/compostos orgânicos com

organocalcogênios [As(III), Se(IV), R–Se–Se–R, Te(IV), R–Te–Te–R] podem interferir na atividade da δ-ALA-D em vários modelos *in vivo* e *in vitro* (Artigo 1). Estudos cristalográficos da δ-ALA-D de leveduras mostram que o Pb(II) e o Hg(II) também são capazes de manter ligações coordenadas com os tiolatos das cisteínas do sítio ativo, em substituição ao Zn(II) (Figura 7-B e 7-C, Artigo 1). De fato, todos esses metais e organocalcogênios possuem centros eletrófilos mais moles quando comparado com o Zn(II) (que é um eletrófilo duro), o que parece explicar a preferência dos tiolatos da δ-ALA-D (e de outras zincoproteínas) por todas essas espécies em detrimento do Zn(II). Com a ligação desses metais, ocorre uma alteração funcional e estrutural na δ-ALA-D, inviabilizando o reconhecimento do substrato ALA pela enzima (Artigos 1 e 2, JAFFE et al., 2001; BELL & VALEE, 2009).

A farmacologia e a toxicologia de disselenetos de diarila, e em especial, do disseleneto de difenila $(PhSe)_2$, tem sido bastante estudada nos últimos anos. No que diz respeito à toxicologia, tem-se observado que o $(PhSe)_2$ inibiu a δ-ALA-D em eritrócitos humanos (CI_{50} = 40 μmol.L^{-1}), fígado e cérebro de ratos (fígado: CI_{50} = 9 μmol.L^{-1}; cérebro: CI_{50} = 4,6 μmolL^{-1}), brânquias de peixe jundiá (*Rhamdia quelen*, CI_{50} = 0,27 mmol.L^{-1}) e em mosca de frutas (*Drosophila melanogaster*) *in vitro* por oxidação de tióis, uma vez que o agente redutor ditiotreitol (DTT) foi capaz de reverter a inibição ocasionada (Tabela 2 do Artigo 1; Artigo 2; SOARES et al., 2004; GOLOMBIESKI et al, 2008). Já no que tange aos estudos *in vivo*, em alguns trabalhos é demonstrada uma diminuição na atividade da δ-ALA-D pela exposição ao $(PhSe)_2$ em roedores com consequente alterações metabólicas (Tabelas 4 e 6 do Artigo 1), enquanto que em outros trabalhos não foram observadas alterações na atividade da enzima em roedores e coelhos (Tabela 3 do Artigo 1). Essas diferenças encontradas podem estar relacionadas à dose administrada, ao modelo animal utilizado, à via de administração e ao tempo de exposição do animal à droga (Artigo 1), o que sugere maiores investigações para buscar uma dose segura com efeito biológico favorável, além de não se descartar totalmente o risco de sua toxicidade como inibidor da δ-ALA-D *in vivo*.

De forma a explicar como ocorreria a oxidação dos tióis catalíticos, Farina e colaboradores (2002) especularam que a inibição da δ-ALA-D de mamíferos poderia ocorrer via oxidação de dois grupos tióis vizinhos localizados no centro ativo da enzima (FARINA et al., 2002; ver Figura 2 do Artigo 2). Nesse esquema proposto, um grupo –SH mais reativo (marcado com um *) deve fazer um ataque nucleofílico em um dos átomos de selênio do disseleneto (passo 1), formando o primeiro selenofenol (PhSeH). Em seguida, seria esperado do –SH vizinho menos reativo atacar o átomo de enxofre do intermediário E–S*-SePh,

formando o segundo selenofenol (PhSeH) e, consequentemente, oxidando os dois tióis vizinhos, fazendo assim com que a enzima seja inibida (Figura 2 do Artigo 2). A partir desta ideia, buscamos métodos de modelagem molecular *in silico* para uma melhor compreensão deste mecanismo de interação. Através do método de *docking* proteína-ligante, buscou-se verificar o modo de afinidade do $(PhSe)_2$ no sítio ativo da δ-ALA-D. De acordo com o modelo de interação da conformação de mais baixa energia livre (ΔG_{bind}) do $(PhSe)_2$ (teoricamente mais estável) demonstrada pelo programa AutoDock Vina, sugere-se que o composto consegue acessar com sucesso o sítio catalítico α/β barril da δ-ALA-D (figura 5 do Artigo 2; Figura 3 do Manuscrito), havendo um posicionamento dos anéis aromáticos do disseleneto voltados para a cadeia lateral de resíduos hidrofóbicos (Tyr, Phe) e positivamente carregados (Lys, Arg), via interações hidrofóbicas e eletrostáticas do tipo empilhamento π-π (grupo fenil do $(PhSe)_2$ e grupo fenil do resíduo Phe208), cátion-π (grupo fenil do $(PhSe)_2$ e cadeia lateral do resíduo Lys199; grupo fenil do $(PhSe)_2$ e cadeia lateral do resíduo Arg209) e C-H…π (grupo fenil do $(PhSe)_2$ e o carbono $C_{\delta 2}$-H do anel aromático do resíduo Phe79) (Figura 4 do Artigo 2). Esse posicionamento dos anéis aromáticos do disseleneto é crucial para permitir a aproximação dos dois átomos de Se da molécula aos tiolatos dos resíduos Cys122, Cys124 e Cys132 ligados ao Zn(II) (Figura 4 do Artigo 2), contribuindo para uma subsequente oxidação desses tiolatos.

Além do $(PhSe)_2$, estudos também têm sido realizados para compreender a atividade biológica de outros análogos com substituintes nas posições para (-OCH_3, -Cℓ) e meta (-CF_3) dos anéis aromáticos do $(PhSe)_2$ em vários modelos experimentais: compostos *bis* (4-clorofenil)disseleneto [(*p*CℓPhSe)$_2$], *bis*(4-metoxifenil)disseleneto [(*p*CH_3OPhSe)$_2$] e *bis* [3-trifluormetil(fenil)] disseleneto [(*m*CF_3PhSe)$_2$] (Artigo 1, Figura 1 do Artigo 2). Estudos *in vitro* e *in vivo* também demonstram que esses compostos podem inibir a atividade da δ-ALA-D em roedores (Tabelas 2 e 4 do Artigo 1). Ao que parece, o acréscimo desses grupamentos não interferem na toxicidade, muito pelo contrário. O composto (*p*CℓPhSe)$_2$, por exemplo, demonstrou uma inibição da atividade da δ-ALA-D de rato maior comparada com o $(PhSe)_2$ (fígado: CI_{50} = 2 μmol.L^{-1} *versus* 9 μmol.L^{-1}; cérebro: CI_{50} = 4,3 μmol.L^{-1} *versus* 4,6 μmolL^{-1}) (Tabela 2 do Artigo 1).

Diante de tais evidências, também buscamos avaliar o modo de interação desses compostos por *docking* molecular. De acordo com os modelos moleculares propostos, os compostos apresentam um modo de interação semelhante ao $(PhSe)_2$ (anéis aromáticos do ligante com interações envolvendo resíduos de Phe, Tyr, Lys e Arg). Além disso, outras interações também devem contribuir para a estabilidade do complexo: ligações de hidrogênio

envolvendo os resíduos Gln225 e Arg221 e o grupo metoxil do composto ($p$$CH_3OPhSe)_2$ e entre dois átomos de flúor de um dos grupos $–CF_3$ do composto ($m$$CF_3OPhSe)_2$ e hidrogênios da cadeia lateral dos resíduos Tyr276 e Gly80 (Figura 4 do Artigo 2). A inclusão de Cℓ (elemento mais eletronegativo) nas posições para dos anéis no composto ($p$$C\ell PhSe)_2$ também contribui para interações eletrostáticas atrativas mais fortes com resíduos carregados positivamente de Lys e Arg do sítio ativo da δ-ALA-D, o que poderia explicar uma maior afinidade desse composto quando comparado com o $(PhSe)_2$ (Figura 4 do Artigo 2).

O estudo de Farina e colaboradores (2002) demonstra ainda que o $(PhSe)_2$ não é capaz de inibir a atividade da δ-ALA-D do pepino (*Cucumis sativus*). Isso se dá pelo fato de que o sítio ativo da δ-ALA-D de plantas, apesar de apresentar os mesmos resíduos hidrofóbicos e positivamente carregados que a δ-ALA-D de animais (Apêndice 1, Figura 1A), não possui em seu sítio catalítico resíduos de cisteína (Cys, C) coordenados a Zn(II), mas resíduos de ácido aspártico (Asp, D) coordenados a Mg(II) (JAFFE, 2003). Essa diferença encontrada na enzima de plantas inviabilizaria uma completa interação dos disselenetos de diarila, tal como ocorre na enzima de animais (Ver Apêndice 1, Artigos 1 e 2, Manuscrito). Kade e Rocha (2010) avaliaram os efeitos do análogo dicolesteroil disseleneto sobre a atividade da δ-ALA-D de camundongo, comparando com o $(PhSe)_2$. De acordo com essa pesquisa, o $(PhSe)_2$ foi capaz de inibir a atividade (como já era esperado), mas o dicolesteroil disselento não alterou a atividade da δ-ALA-D nas concentrações testadas (KADE & ROCHA, 2010). Levando-se em consideração que o colesteroil é um grupo muito volumoso, podemos sugerir que compostos análogos ao disseleneto com substituintes muito volumosos seriam impossibilitados em acessar o sítio catalítico da δ-ALA-D para promover oxidação dos tióis catalíticos.

Apesar de o *docking* ter demonstrado ser um método eficaz para predizer o modo de interação entre os diaril disselenetos e a δ-ALA-D (Artigo 2), o mesmo ainda não é adequado para dar informações mais precisas quanto às contribuições individuais de cada resíduo de aminoácido no sítio ativo. Tal informação seria crucial para compreender melhor os mecanismos de interação entre ligante e proteína, além de contribuir com informações para o planejamento de drogas com baixa afinidade para a δ-ALA-D. Diante disso, partimos para métodos de bioquímica quântica, que conseguem descrever melhor a interação considerando a densidade eletrônica do sistema. No entanto, como a aplicação desses métodos para sistemas muito grandes (como é o caso de proteínas) ainda é computacionalmente inviável, resolveu-se adotar o método de fragmentação MFCC como forma de avaliar as energias de interação individuais, que tem tido relativa correlação com dados experimentais (Manuscrito). De acordo com esse método, foi possível observar que interações entre o $(PhSe)_2$ e resíduos de

aminoácidos do sítio de ligação podem acontecer até um raio de 8,5 Å partindo do centroide do ligante. Além disso, pôde-se confirmar que os resíduos Lys199, Lys252 (ambos essenciais para a formação de base de Schiff com o substrato ALA), Phe208, Phe79, além das Cys122, Cys124 e Cys132 complexas com Zn(II), apresentam as maiores energias de interação com o $(PhSe)_2$, exercendo uma importante força eletrostática atrativa para que ocorra a estabilidade do complexo. Além desses resíduos, outros próximos também foram identificados exercendo forças eletrostáticas atrativas consideráveis (Pro125, Arg221, Asp120, Tyr126, Tyr205 e Gln225) com os anéis aromáticos do ligante.

5 CONCLUSÕES

Com a utilização de métodos *in silico* (*docking* molecular e cálculos quânticos) foi possível, pela primeira vez, propor um modelo molecular correlacionado com dados experimentais, já que o mesmo é capaz de explicar o mecanismo de oxidação dos tióis catalíticos da δ-ALA-D por disselenetos de diarila, com as seguintes observações:

- O modelo molecular sugere que a interação entre o $(PhSe)_2$ e a enzima δ-ALA-D se dá através de uma forte energia de interação atrativa envolvendo os anéis aromáticos do $(PhSe)_2$ e resíduos hidrofóbicos (Phe208, Phe79) e positivamente carregados (Lys 199 e Lys252), devido a interações eletrostáticas π-π, cátion-π e CH...π;

- A interação dos anéis aromáticos do $(PhSe)_2$ com resíduos hidrofóbicos permite posicionar os átomos de Selênio do composto próximo aos resíduos Cys122, Cys124 e Cys132 complexados com Zn(II) (que também exercem, conjuntamente, uma energia de interação atrativa). Essa aproximação permitirá uma subsequente oxidação dos tiolatos.

- De acordo com o método MFCC, o modelo molecular do complexo sugere que o $(PhSe)_2$ é capaz de interagir com os resíduos localizados até a uma distância de 8,5 Å partindo do seu centroide.

- Os disselenetos de diarila $(pClPhSe)_2$, $(pCH_3OPhSe)_2$ e $(mCF_3PhSe)_2$ também conseguem acessar facilmente o sítio ativo α/β barril da δ-ALA-D, de forma semelhante ao $(PhSe)_2$.

- O caráter mais eletronegativo e a presença de receptores de ligação de hidrogênio nos substituíntes também parecem contribuir para uma maior afinidade no sítio ativo da δ-ALA-D.

- Conjuntamente, a presença dos dois anéis aromáticos nos disselenetos de diarila e a flexibilidade da ligação C-Se-Se-C dos disselenetos de diarila parecem ser cruciais para que haja forte interação com resíduos hidrofóbicos a enzima δ-ALA-D, abrindo uma nova perspectiva quanto a trabalhos futuros envolvendo o desenvolvimento de novos organocalcogênios com menor toxicidade frente à inibição da δ-ALA-D.

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APÊNDICE

APÊNDICE A – ALINHAMENTO MÚLTIPLO DE SEQUÊNCIAS DE AMINOÁCIDOS DA δ-ALA-D DE DIFERENTES ORGANISMOS

Figura 1: Alinhamento múltiplo das sequências de aminoácidos da δ-ALA-D de diferentes organismos usando o programa ClustalW. Em verde, resíduos que estão posicionados até 4 Å do ligante $(PhSe)_2$ no *docking* molecular com a enzima δ-ALA-D humana. Em amarelo, resíduos de cisteína que interagem com o íon Zn(II), de acordo com o cristal PDB 1E51. Em cinza, resíduos específicos da δ-ALA-D de plantas, não encontrados na enzima dos animais. Os dados sugerem que o (PhSe)2 tem maior afinidade com a δ-ALA-D de animais (com atividade inibitória de modo semelhante), mas não de plantas, corroborando com dados experimentais (Farina et al., 2002).

ANEXO

ANEXO A – CAPA DA REVISTA *TOXICOLOGY RESEARCH*

Toxicology Research

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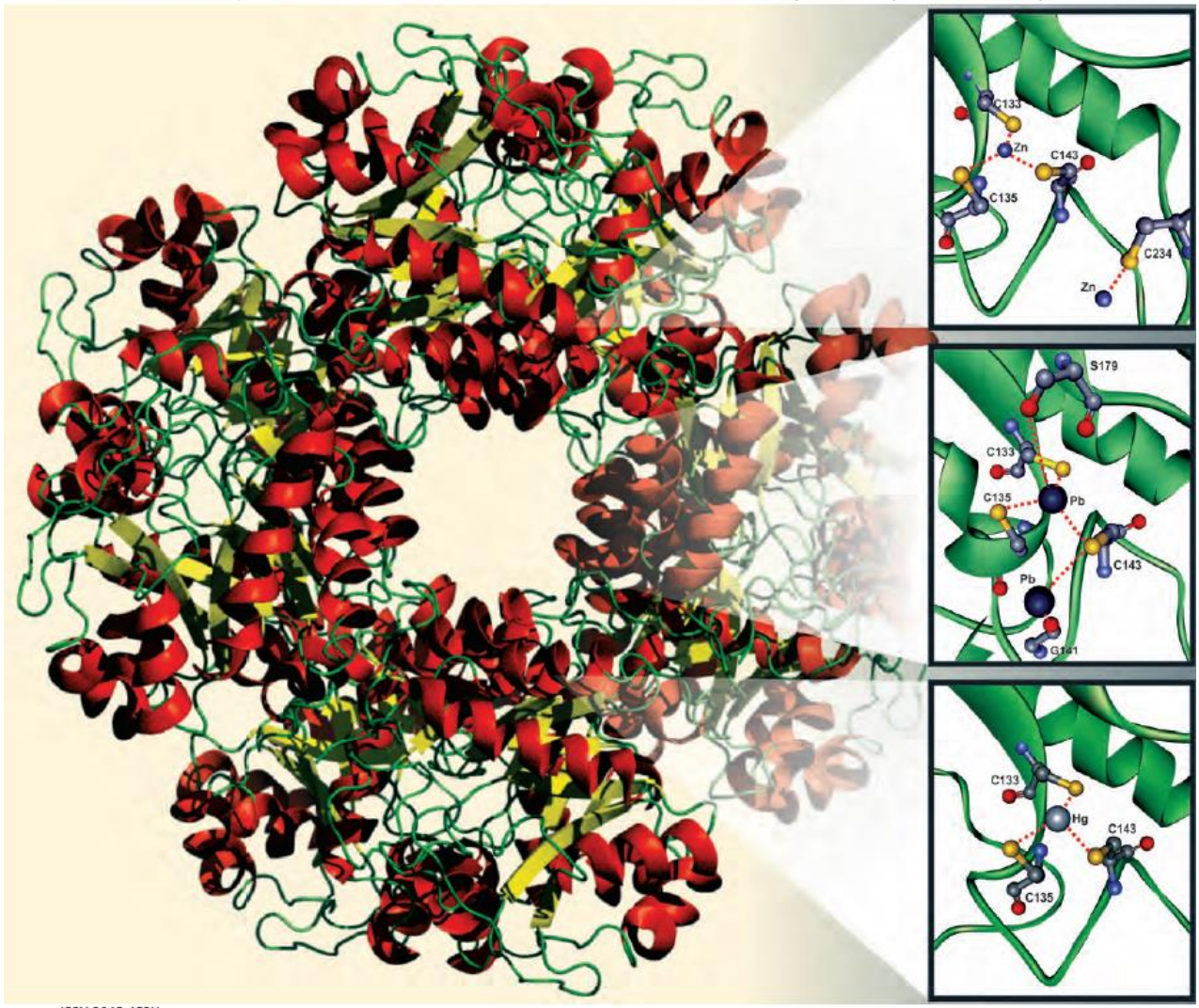

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