**Universidade de Brasília**
**Faculdade de Medicina**
**Laboratório da Interação Parasito-Hospedeiro**

# Propriedades Termodinâmicas da Prolil Oligopeptidase de *Trypanosoma brucei*

**MEIRE MARIA DE LIMA**

**Orientador: Prof. Dr. Jaime Martins de Santana**
**Co-orientadora: Prof[a]. Dr[a]. Sônia Maria de Freitas**

Tese apresentada ao programa de
Pós-graduação em Patologia Molecular da
Universidade de Brasília como requisito parcial
à obtenção do título de Doutor em Patologia Molecular.

**- Brasília, 2008 –**

Trabalho desenvolvido no Laboratório da Interação Parasito-Hospedeiro, Universidade de Brasília, sob a orientação do Prof. Jaime Santana, e no Laboratório Nacional de Luz Síncrotron, Campinas, sob co-orientação da Profª. Sônia Freitas. Apoio financeiro de CAPES, CNPq, FAPDF, FINEP e ABTLuS.

**“Only those who attempt the absurd**
**will achieve the impossible.”**
**M. C. Escher**

# AGRADECIMENTOS

Ao Professor Jaime Santana por ter aceitado o desafio de ‘ensinar a caminhar’, pelo exemplo de retidão e bom senso e por provar que é possível a pesquisa de qualidade no Brasil de hoje;

À Professora Sônia Freitas pela paciência e infinita disposição em ensinar e por fazer a biofísica passar a existir para mim;

-Professores Jaime e Sônia, se eu fosse professora, é como vocês que eu gostaria de ser!

Ao Javier Medrano pela generosidade com que dispôs do seu tempo e por nunca ter-se recusado a ensinar e ajudar;

Ao João Alexandre por, dentre outras coisas, ir ao laboratório com enxaqueca olhar um cristal (era de sal, que pena!);

Aos colegas do Laboratório de Chagas e do Laboratório da Interação Parasito-Hospedeiro pela cumplicidade e ajuda. Em especial, agradeço a Carol, David, Ellen, Flavita, Glorita, Iza, Mari, Nadjar, Tecris e Rúbens – toda a diferença vocês fazem no ambiente de trabalho;

A Cássia, Geraldo, Miguel e Márcia pelas conversas, cafés, sorrisos e ‘socorros’;

A Daiana, Dong-sun, Madhu Sharma, Pierre Nioche, Sudha e Raman por fazerem inesquecível a estada na The University of Texas. Thank y’all!

À Renata por me trazer para o doutorado, pelo encorajamento e pela cumplicidade. Você é uma boa amiga!

`A família Lima por fazer a frase ‘edificada sobre a rocha’ ter sentido;

Ao Paulo Chuc por tornar a caminhada mais suave;

Ao Benjamin Chuc, sumidouro de ansiedades.

SOU MUITO GRATA A TODOS.

# LISTA DE ABREVIATURAS

| | |
|---|---|
| [θ] | Eliticidade média por resíduo |
| °C | Grau centígrado |
| CMC | Concentração micelar crítica |
| CD | Dicroísmo circular |
| cm | centímetro |
| CTAB | Brometo de hexadecil-trimetilamonia |
| Da | Dalton |
| DTT | Ditiotreitol |
| g | Grama |
| GuHCl | Hidrocloreto de guanidina |
| HEPES | Ácido (2-hidroxietil)-piperazina etanosulfônico |
| His | Histidina |
| IPTG | Isopropil-β-D-tiogalactopiranosídeo |
| M | Molar |
| NMR | Ressonância magnética nuclear |
| SDS-PAGE | Eletroforese em gel de poliacrilamida contendo dodecil sulfato de sódio |
| Tris | Tris-hidroximetil-etano |
| Trp | Triptofano |
| UV | Ultravioleta |
| v/v | Razão volume/volume |
| $\Delta G$ | Variação da energia livre de Gibbs |
| $\Delta H$ | Variação da entalpia |
| $\Delta S$ | Variação da entropia |

# ABREVIATURA DOS AMINOÁCIDOS

| | |
|---|---|
| A | Alanina |
| C | Cisteína |
| D | Ácido aspártico |
| E | Ácido glutâmico |
| F | Fenilalanina |
| G | Glicina |
| H | Histidina |
| I | Isoleucina |
| K | Lisina |
| L | Leucina |
| M | Metionina |
| N | Asparagina |
| P | Prolina |
| Q | Glutamina |
| R | Arginina |
| S | Serina |
| T | Treonina |
| V | Valina |
| W | Triptofano |
| Y | Tirosina |

# ÍNDICE

# RESUMO

*Trypanosoma brucei* secreta uma prolil oligopeptidase (POPTb) que parece estar envolvida na patogênese da doença do sono. Essa enzima é capaz de hidrolizar hormônios peptídicos contendo resíduos de prolina e considerada um potencial alvo para o desenvolvimento de drogas contra a patologia. Como a estrutura tridimensional da POPTb ainda não foi resolvida, dicroísmo circular e espectroscopia de fluorescência foram utilizados para estudo da estrutura e estabilidade da enzima bem como para a identificação de aditivos que possam ser utilizados nos experimentos de cristalização e na estocagem da proteína. Parâmetros termodinâmicos e estruturais foram calculados a partir de experimentos de desnaturação térmica e química e de estudos de atenuação da fluorescência. Cloreto de guanidina (GuHCl) promoveu a desnaturação da proteína em concentrações quase 30% menores que uréia. Divergência encontrada nos valores de $\Delta G$ determinados a partir de experimentos de desnaturação química e térmica sugere a presença de intermediários no processo de desdobramento da proteína. Sugere, também, maior estabilidade estrutural. Sorbitol se mostrou capaz de estabilizar a POPTb enquanto brometo de hexadecil-trimetilamônia (CTAB), na concentração de 0,1 mM, provocou o efeito oposto levando à quase total inatividade da enzima. A ação do ditiotreitol (DTT) é pH-dependente, reduzindo a estabilidade da enzima em pH neutro e aumentando-a em pH alkalino. Experimetos de atenuação da fluorescência mostraram dependência do pH e heterogeneidade no ambiente dos triptofanos com pelo menos um resíduo desse aminoácido próximo ao sítio ativo. A enzima parece estar menos sujeita a alterações conformacionais em pH 7.5. A elevação da temperatura resultou na redução da concentração micelar crítica (cmc) do CTAB.

# ABSTRACT

*Trypanosoma brucei* secrets a prolyl oligopeptidase (POPTb) which is possibly involved in the pathogenesis of sleeping sickness. It is able to hydrolyze proline-containing peptide hormones and is therefore considered a good target for the development of drugs to treat the disease. As the crystal structure of POPTb has not been solved yet, we used circular dichroism and fluorescence spectroscopy to gain some knowledge on the enzyme structure, stability and on additive effects for both crystallization assays and storage. Thermodynamic and structural parameters were calculated from thermal and chemical denaturation studies as well as from fluorescence quenching experiments, respectively. The concentration of guanidine hydrochloride (GuHCl) that led to protein denaturation was nearly 30% that of urea needed to reach the same effect. Divergence in the $\Delta G$ values derived from chemical and thermal unfolding assays suggests the presence of intermediate states in the process. It also suggests higher structural stability. Sorbitol increases enzyme stability and 0.1 mM cetyl trimethyl ammonium bromide (CTAB) decreases it almost halting enzyme activity. Ditiotreitol (DTT), however, has the former effect in alkaline pH and the latter effect in neutral pH. Fluorescence quenching experiments show dependence on pH and indicate heterogeneity in the environment of the surface-accessible tryptophan residues with at least one such residue near the enzyme active site. POPTb molecule seems to be less subjected to conformational changes at pH 7.5. The increase in temperature lowers CTAB critical micelle concentration (cmc).

# INTRODUÇÃO

**“Wonder is what sets us apart from other life forms. No other species wonders about the meaning of existence or the complexity of the universe or themselves.”**
**Herbert W. Boyer**

# INTRODUÇÃO

## Doença do Sono

Também conhecida como Tripanossomíase Africana Humana, é causada pelos protozoários parasitas *Trypanosoma brucei gambiense* no oeste e centro da África e *Trypanosoma brucei rhodesiense* no leste e sul. A principal forma de transmissão é por meio da picada de moscas tsé-tsé (*Glossina* spp) (Fig. 1) contaminadas com os parasitos, mas também pode se dar através de sangue infectado ou da placenta (transmissão congênita). Como as moscas tsé-tsé são nativas do continente africano e encontradas apenas em regiões sub-saharianas, é nestes locais que a doença se manifesta. Mostra-se essencial a presença simultaneamente do tripanossoma, do vetor e do hospedeiro para a transmissão da doença do sono (WHO, 2006).

**Figura 1**. *Glossina* spp. (mosca tsé-tsé).
(Who/TDR).

*Trypanosoma brucei brucei* infecta o gado na África causando a patologia conhecida como nagana. Os animais também não resistem à infecção e morrem, o que contribui para o aumento dos problemas enconômicos nas áreas afetadas (Shiflett *et alii*, 2006). Além de humanos e bovinos, o *T. brucei* infecta vários mamíferos. Os animais podem ser hospedeiros dos patógenos humanos e a importância desses hospedeiros para manutenção da doença do sono causada pelo *T. b. rhodesiense* é indiscutível. Há

controvérsia, no entanto, quanto ao papel de animais como reservatórios do *T. b. gambiense* (Brun & Balmer, 2006).

Em meados dos anos 60, a doença havia praticamente desaparecido graças a anos de intensos esforços no sentido de controlá-la. A independência dos países africanos nos quais a doença é endêmica trouxe consigo a falta de recursos que, aliada à baixa incidência naquele momento, levou a doença do sono a não ser considerada prioridade. As conseqüências podem ser avaliadas por meio de estimativas atuais, as quais dão conta de aproximadamente 400.000 casos a cada ano, além de 60 milhões de pessoas sob risco de contraírem a moléstia (Hellemond & Tielens, 2006), invariavelmente fatal se não tratada. As drogas disponíveis contra a patologia apresentam características que dificultam o tratamento, tais como toxicidade, administração parenteral, estoques insuficientes e incidência crescente de tratamentos malsucedidos (Barrett *et alii*, 2007).

## Ciclo de Vida do *Trypanosoma brucei*

O ciclo de vida do *T. brucei* é complexo e envolve o desenvolvimento e a multiplicação de formas do parasito no hospedeiro mamífero e no inseto vetor. O protozoário vive exclusivamente como parasita extracelular e sofre transformações morfológicas e metabólicas – por exemplo, adaptação do metabolismo energético e de lípides – como forma de enfrentar os diferentes ambientes com que se depara durante seu ciclo evolutivo (Lee *et alii*, 2006; Hughes *et alii*, 2007).

Durante repasto sanguíneo, moscas tsé-tsé injetam em hospedeiro mamífero formas tripomastigotas metacíclicas de *T. brucei* presentes na saliva. Os parasitas entram no sistema linfático e depois na corrente sanguínea. As formas metacíclicas se transformam em tripomastigotas sanguíneas, alcançam outros fluidos corporais - tais como a linfa e o líquor -, e continuam a se replicar. A replicação do *T. brucei* se dá por fissão binária.

A mosca tsé-tsé se infecta ao ingerir formas tripomatigotas sanguíneas durante repasto em hospedeiro mamífero contaminado. Essas formas se transformam em tripomastigotas procíclicos no intestino médio do vetor onde se multiplicam. As formas procíclicas deixam o intestino médio e se transformam em epimastigotas que alcançam as

glândulas salivares. Neste local, continuam a se multiplicar e se transformam em tripomastigotas metacíclicos. O ciclo se completa em aproximadamente 3 semanas quando a mosca vetor terá formas tripomastigotas metacíclicas na saliva e poderá, durante alimentação, infectar outro mamífero (Fig.2).

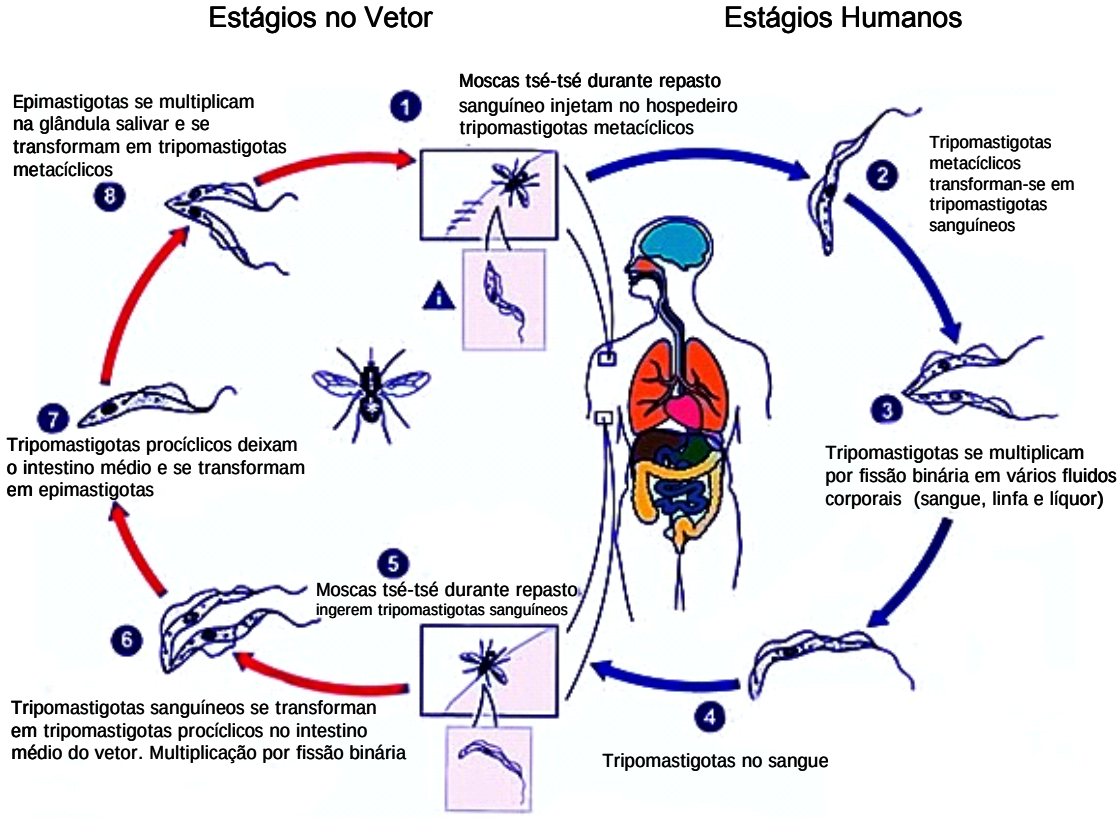


**Figura 2**. Ciclo de vida do *Trypanosona brucei.*
(Adaptado de http://www.dpd.ccd.gov/dpdx/HTML/TrypanosomiasisAfrican.htm)

## A Doença

*T. brucei gambiense* é responsável por 90% dos casos relatados e causa infecção crônica que pode durar vários anos. *T. brucei rhodesiense* é responsável por menos de 10% dos casos relatados, causa infecção aguda de desenvolvimento rápido e invade o sistema nervoso central. Pode levar à morte em semanas ou meses. Os dois tipos da doença levam à morte pacientes não tratados.

Nos estágios iniciais, os parasitas se multiplicam na corrente sanguínea e no sistema linfático levando basicamente ao característico inchaço dos linfonodos cervicais. A segunda fase se inicia no momento em que o *T. brucei* atravessa a barreira hematoencefálica e invade o sistema nervoso central. Desencadeia os sintomas neurológicos e sinais característicos, os quais incluem alteração do estado mental, desordens sensoriais e problemas de coordenação. É nesse estágio que a moléstia causa a desordem da qual se origina o seu nome: alteração do ciclo circadiano de vigília e sono. Alterações endócrinas, cardiovasculares e renais importantes também ocorrem (Kennedy, 2006).

A progressão natural da doença não-tratada passa por sonolência, coma e morte. Entretanto, a doença do sono é de difícil tratamento, especialmente após o tripanossoma ter cruzado a barreira hematoencefálica. Além disso, esses parasitos são capazes de evadir o sistema imune do hospedeiro graças ao enorme potencial para variação antigênica que apresentam (Lee *et alii*, 2006).

O controle da doença do sono baseia-se na redução dos reservatórios de infecção por meio de diagnóstico precoce e controle do inseto vetor.

## Tratamento

O tratamento da doença do sono é estágio-dependente. Assim como em outras tantas patologias, quanto mais rápido o diagnóstico, melhores os prognósticos. As drogas usadas na primeira fase da doença são menos tóxicas, de mais fácil administração e mais eficazes. São elas pentamidina e suramina para tratamento de infecção pelo *T. b. gambiense* e *T. b. rhodesiense*, respectivamente (Barrett *et alii*, 2007).

Já na segunda fase, o tratamento depende de drogas que consigam atravessar a barreira hematoencefálica para atingirem o parasita. Essas drogas são tóxicas, de difícil administração e podem mesmo matar. Um exemplo é o melarsoprol, usado nas duas formas da doença e que causa encefalopatia como efeito colateral. Estima-se que entre 3% e 10% dos pacientes não resistam ao tratamento (WHO, 2006). Ademais, resistência a esse medicamento tem sido observada particularmente na África central. A outra opção de tratamento disponível é eflornitina que, embora menos tóxica que melarsoprol, só tem ação contra o *T. b. gambiense* (Barrett *et alii*, 2007).

Essas drogas foram descobertas entre 1921 e 1949, à exceção da eflornitina que o foi em 1990. É evidente a necessidade do desenvolvimento de novos fármacos que possam ser usados no tratamento da doença do sono em qualquer de suas formas, além de melhoria do acesso às atualmente disponíveis (WHO, 2006).

## Serino Proteases

Serino proteases são uma classe de peptidases caracterizadas pela presença de um resíduo de serina no sítio ativo da enzima. Esse resíduo possui uma hidroxila que participa da reação de catálise e ligação ao substrato. As famílias que compõem essa classe de proteases incluem a da quimotripsina (S1), da subtilisina (S8) e da prolil oligopeptidase (S9). Todas essas enzimas são inibidas por diisopropilfluorofosfato (DFP), muitas por fenilmetilsulfonil fluoreto (PMSF) e algumas por n-α-tosil-L-lisina clorometil cetona (TLCK) e n-α-tosil-L-fenilalanina clorometil cetona (TPCK). Os dois últimos alquilam resíduos de histidina do sítio ativo e são, assim, inibidores eficazes. As serino proteases participam de vários processos biológicos tais como coagulação sanguínea, imunidade, inflamação e digestão.

## Família Prolil Oligopeptidase ou S9

Estão incluídas nessa família de serino proteases as prolil oligopeptidases propriamente ditas (POP, EC 3.4.21.26), a dipeptidil peptidase IV (DPPIV, EC 3.4.14.5), a acilaminoacil peptidase (ACPH, EC 3.4.19.1) e a oligopeptidase B (OB, EC 3.4.21.83) (Rawlings *et alii*, 1991). As especificidades para substrato são diferentes entre as enzimas dessa família, já que as POP hidrolizam peptídeos do lado carboxílico de resíduos de prolina, DPPIV libera dipeptídeos nos quais o penúltimo aminoácido é prolina, OB cliva peptídeos em lisina e arginina e ACPH remove aminoácidos N-acetilados de peptídeos bloqueados (Venäläinen *et alii*, 2006).

Os membros dessa família distinguem-se das serino proteases clássicas – tripsina e subtilisina – no que se refere à especificidade por substratos peptídicos, à topologia da

tríade catalítica e à localização dessa na porção C-terminal da proteína (Venäläinen *et alii*, 2006). Além disso, são muito maiores (aproximadamente 80 kDa) que as clássicas (25-30 kDa) (Szeltner *et alii*, 2004).

POPs foram purificadas de procariotos e eucariotos, apresentam alta homologia e resíduos de triptofano e cisteína conservados (Fig.3). A estrutura tridimensional da POP suína na ausência e presença de inibidor já foi resolvida (Fülop, 1998; PDB 1qfm e 1qfs) e mostra que a proteína apresenta um domínio catalítico e um chamado ß-propeller. O primeiro é formado pelas porções N e C-terminal da cadeia polipeptídica, engloba a tríade catalítica e exibe o arranjo clássico α/ß hidrolase. O domínio ß-propeller é formado por folhas ß paralelas que se dispõem em um total de 7 pás. Elas se colocam radialmente em torno de um túnel central. O domínio ß-propeller clássico apresenta um 'velcro' unindo a primeira e a última pás, o qual é formado por pontes de hidrogênio e dissulfeto. As POPs, entretanto, possuem um domínio ß-propeller peculiar já que apresenta topologia aberta e interações hidrofóbicas relativamente fracas estabilizando as pás (Fülop, 1998).

A ausência do 'velcro' permite uma abertura parcial do domínio ß-propeller e aumento do túnel central, o qual seria o meio de acesso do substrato à tríade catalítica localizada na interface dos dois domínios. O túnel funcionaria como um filtro permitindo a passagem apenas de substratos com até 30 aminoácidos, o que protegeria de hidrólise proteínas e peptídeos maiores (Fülop, 1998, 2000). No entanto, Szeltner *et alii* (2004) mostraram a necessidade de movimentos consonantes entre o domínio α/ß hidrolase e o ß-propeller para atividade da enzima. A especificidade para peptídeos menores é considera uma das características mais marcantes das POPs.

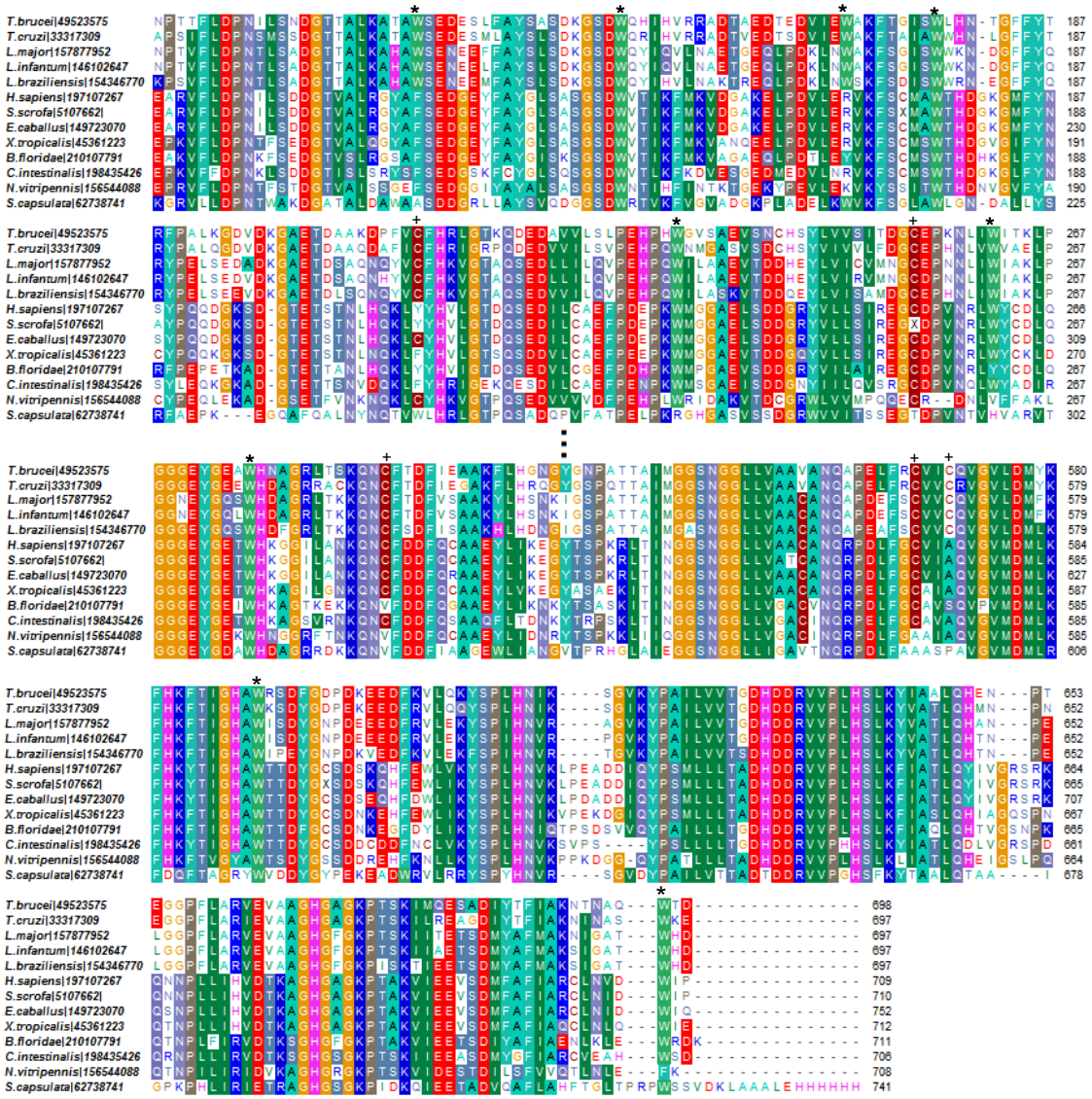


**Figura 3**. Alinhamento múltiplo de sequências de POPs de vários organismos por meio de ClustalW. Resíduos de triptofanos estão marcados com asterisco (*) e de cisteína com cruz (+).

Nosso grupo demosntrou, todavia, a capacidade da POP de *Trypanosoma cruzi* (POPTc80) de hidrolizar os componentes da matriz extracelular colágeno I e IV e fibronectina (Santana *et alii*, 1997). Tal fato demonstrou a necessidade de uma rota alternativa de acesso ao sítio catalítico, uma vez que a alta massa molecular daquelas proteínas as impediria de adentrarem o túnel do ß-propeller. A nossa proposta, baseada em

experimentos de 'docking', foi de que o substrato induziria uma abertura entre os dois domínios da enzima permitindo, assim, que o substrato alcançasse o sítio ativo (Fig. 4) (Bastos *et alii*, 2005). Shan *et alii* (2005) validaram nossa proposta ao resolverem a estrutura da POP de *Sphingomonas capsulata* em configuração aberta. Ainda, a introdução da ligação dissulfeto unindo os dois domínios resulta em inativação da enzima quando em ambiente oxidado; a POPTc80 recupera a atividade após redução (nossos dados não publicados).

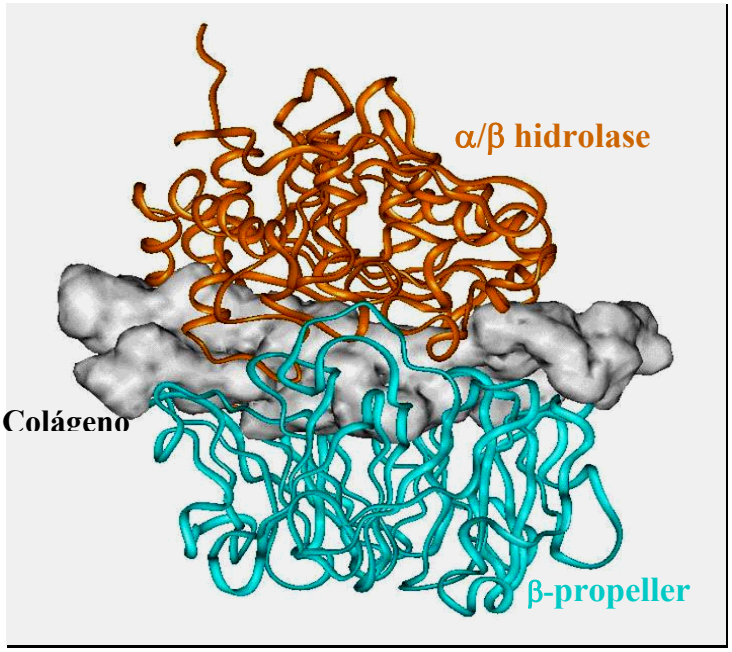


**Figura 4.** 'Docking'da tripla hélice do colágeno com a POPTc80 (Bastos *et alii*, 2005).

Demonstramos, ainda, a importância da POPTc80 para o *T. cruzi* uma vez que inibidores específicos contra essa enzima são capazes de impedir a entrada do parasito na célula mamífera hospedeira não-fagocítica, passo essencial no seu ciclo de vida. (Grellier *et alii*, 2001; Bastos *et alii*, 2005).

Diferentemente da maior parte das proteases, POPs possuem a importante característica de serem capazes de clivar ligações peptídicas na região C-terminal de resíduos de prolina (Brandt *et alii*, 2005). Tal atributo, aliado ao fato dos resíduos de prolina estarem presentes em muitos peptídeos biologicamente ativos, sugere um possível envolvimento de POPs na modulação dos níveis de peptídeos neuronais e hormônios

contendo tal aminoácido bem como eleva o interesse dessas enzimas como alvos terapêuticos.

Vários substratos de POPs já foram identificados, dentre os quais hormônio liberador de tirotropina (TRH), hormônio liberador de gonadotropina (GnRH), angiotensinas e substância P. No cérebro humano, as atividades mais elevadas de POP são encontradas em áreas envolvidas nos processos de aprendizado e memória (Irazusta *et alii*, 2002). Essas proteases foram implicadas em várias patologias tais como depressão (Polgár, 2002), Alzheimer's (Kowall et alii, 1991) e amnésia (Yashimoto *et alii*, 1987). Estudos com inibidores de POPs descrevem uma relação inversa entre concentração da enzima e capacidade cognitiva (Morain *et alii*, 2002). Recentemente, foi postulada a possibilidade do uso terapêutico de POP no tratamento da doença celíaca. A enzima degradaria os peptídeos ricos em prolina presentes no intestino dos pacientes portadores da patologia (Shan *et alii*, 2002; Hausch *et alii*, 2002). Apesar de todas as indicações, o papel biológico das POPs ainda é alvo de controvérsia, já que *in vivo* os sinais de que a POP é realmente a responsável pelo metabolismo de qualquer dos peptídeos propostos é limitada. Estudos termodinâmicos com POPs podem acrescentar dados que ajudem na compreensão do funcionamento dessa família.

Como a POPTc80 apresenta características de fatores de virulência, nosso grupo caracterizou a proteína homóloga secretada pelo *T. brucei* e observou que elas compartilham 77% de identidade (Fig. 3). Assim como a POPTc80, a POPTb é capaz de hidrolizar colágeno e tem atividade ótima em pH 8.0, a qual é aumentada por DTT. POPTb é sensível aos inibidores irreversíveis da POPTc80, os quais bloqueiam o crescimento das formas procíclicas e sanguíneas de *T. brucei* sugerindo o envolvimento dessa protease no desenvolvimento do parasito (Bastos, tese de doutorado).

POPTb é liberada no plasma de camundongos infectados por T. brucei permanecendo ativa ao longo da parasitemia. Essa enzima hidroliza peptídeos hormonais humanos como bradicinina, ß-endorfina e neurotensina e parece estar envolvida na patogênese da doença do sono (nossos dados não publicados). Por todas essas característica, a POPTb pode ser considerada uma potencial candidata a alvo de drogas.

Neste trabalho, estudamos o comportamento estrutural e a estabilidade da POPTb em diferentes pHs e temperaturas bem como na presença e ausência de aditivos e desnaturantes. Empregamos, com esse fim, técnicas espectroscópicas tais como dicroísmo circular (CD) e fluorescência, cujos fundamentos básicos descrevemos a seguir.

## Dicroísmo Circular

Uma proteína terá atividade biológica se, e somente se, adotar sua estrutura tridimensional correta. Um dos maiores desafios atuais é entender como uma seqüência linear de aminoácidos traz informações para que a estrutura nativa ou conformação ativa da proteína seja atingida. Igualmente desafiante é entender o mecanismo através do qual o dobramento rápido e eficiente da cadeia polipeptídica se dá. A relevância clínica de conhecer tal mecanismo pode ser ilustrada pelo fato de várias doenças estarem ligadas ao dobramento incorreto de proteínas. A doença da 'vaca louca' e Alzheimer são exemplos (Nandi *et alii*, 2002).

A estrutura tridimensional de muitas proteínas já foi resolvida, por meio de cristalografia de raios X e ressonância nuclear magnética (NMR), fornecendo dados estruturais importantes. Entretanto, há a necessidade de estudos estruturais nas condições em que as proteínas exercem sua função (geralmente em solução) além de dados sobre as mudanças estruturais que ocorrem nas proteínas em determinadas circunstâncias – variadas temperaturas, pHs e presença/ausência de ligantes -, já que normalmente são essenciais para a função biológica das proteínas (Kelly *et alii*, 2005).

Dicroísmo circular é considerado uma técnica estrutural importante para tais estudos e tem em sua base o uso da luz circularmente polarizada. Ela pode ser entendida como formada por dois componentes circularmente polarizados de igual magnitude e que giram, um no sentido horário e outro no anti-horário. Se após passarem pela amostra os dois componentes forem absorvidos em quantidades diferentes, a radiação resultante deixa de ser circular e passara a ter polarização elíptica. O aparelho de CD mede a diferença na absorção dos componentes e o espectro de CD é obtido quando a elipcidade é medida em função do comprimento de onda (Kelly *et alii*, 2005).

Um sinal de CD será observado quando um cromóforo for quiral (opticamente ativo) na medida em que: a) é naturalmente quiral devido à sua estrutura; b) está covalentemente ligado a um centro quiral na molécula ou; c) foi colocado em uma ambiente assimétrico devido à estrutura tridimensional adotada pela molécula. Os cromóforos de interesse em proteínas incluem as ligações peptídicas (absorvem abaixo de 240 nm), a cadeia lateral dos aminoácidos aromáticos (absorção entre 260 e 320 nm) e pontes dissulfeto (absorção em torno de 260 nm). Ainda, co-fatores (heme, flavinas) absorvem em diferentes comprimentos de onda e ligantes não quirais podem induzir sinal

de CD ao adquirirem quiralidade como resultado de sua ligação em um ambiente assimétrico tal qual o fornecido por proteínas (Kelly & Price, 1997).

## Aplicações

O estudo de proteínas usando a técnica de CD pode trazer informações sobre:

### 1. Composição da estrutura secundária

O uso da radiação ultravioleta (UV) na faixa distante do espectro (180-240 nm) permite estimar quantitativamente o conteúdo das diferentes formas de estruturas secundárias presentes em proteínas. Isso porque cada tipo de estrutura origina um espectro de CD característico. Os dados assim obtidos são analisados por comparação com bancos de dados formados por espectros de CD de proteínas com vários arranjos de estruturas secundárias e cujas estruturas tridimensionais tenham sido resolvidas por cristalografia de raios X (Kelly *et alii*, 2005).

### 2. Caracterização da estrutura terciária

O espectro de CD na região do UV-próximo (260-320 nm) é originado pelos aminoácidos aromáticos que tendem a absorver a energia da radiação em comprimentos de onda distintos. Triptofanos tem pico máximo próximo a 290 nm, tirosinas entre 275 e 282 nm e fenilalaninas entre 255 e 270 nm. O formato e a magnitude do espectro nessa região do UV depende do número e mobilidade de cada tipo de aromático na proteína, do ambiente em que se encontram além de suas disposições espaciais.

O espectro de CD no UV-próximo fornece dados sobre a estrutura terciária da proteína objeto de estudo que podem ser usados para comparar tipos mutantes e selvagens das proteínas. Além disso, pode fornecer indicações importantes da existência de intermediários como “molten globule” no processo de enovelamento das proteínas (Price, 2000).

### 3. Mudanças conformacionais

O mecanismo de ação e regulação da atividade biológica requer a associação de ligantes que promove alterações estruturais nas proteínas. Tais mudanças podem ser detectadas por meio de CD em diferentes regiões do UV. CD pode ainda ser usado para determinar a faixa de concentração do ligante na qual as modificações ocorrem bem como a extensão dessas. Ademais, CD é especialmente importante para o estudo de peptídeos e proteínas cujas estruturas tridimensionais ainda não foram resolvidas ou nos quais o uso da cristalografia de raios X é impraticável, a exemplo da mudança entre arranjo de α-hélice e folhas ß verificado nos prions (Hope *et alii*, 1996).

## 4. Enovelamento de proteínas

Os métodos de CD chamados fluxo interrompido ou fluxo contínuo podem ser usados para acompanhar mudanças conformacionais que ocorrem durante a aquisição da estrutura nativa, pois detectam alterações na escala de milisegundos. Medindo a taxa de aquisição de estrutura secundária e terciária, estudos sugerem que proteínas de até 100 resíduos de aminoácidos dobram-se rapidamente seguindo um mecanismo de dois estados no qual intermediários são indetectáveis. O enovelamento de proteínas maiores, entretanto, normalmente envolve múltiplos estágios. Nos estágios iniciais, grandes quantidades de estrutura secundária semelhante à nativa são adquiridas antes da formação dos contactos presentes na estrutura terciária da proteína originando intermediários conhecidos como "moltem globule" (Kelly *et alii*, 2005).

## 5. CD versus Cristalografia de Raios X e NMR

Embora não forneça informações em nível de resolução atômica, CD é uma técnica que demanda quantidades bem menores de amostra e menos tempo de execução que cristalografia de raios X e NMR. CD pode explorar estruturas de proteínas em inúmeras condições experimentais bem como avaliar a estabilidade e a estrutura de uma proteína nas condições a serem utilizadas nos experimentos de NMR, tais como altas concentrações da proteína, pH levemente ácido, altas temperaturas, tempo de aquisição prolongado. Similarmente, CD pode ser usado para explorar a estrutura de uma proteína nas condições próximas daquelas usadas para cristalografia e compará-la com aquela nas condições que se imagina sejam as mais relevantes fisiologicamente.

## Espectroscopia de Fluorescência

Espectroscopia de fluorescência é técnica amplamente usada para estudos em nível molecular que ajudam na compreensão da função biológica das proteínas. Pode também ser usada para estudar as propriedades estruturais e dinâmicas das proteínas que estejam diretamente relacionadas às funções biológicas que desempenham, tais como ligação, biocatálise, transporte transmembrânico e motilidade muscular.

Todos os compostos químicos absorvem energia que causa excitação dos elétrons na molécula, tais como aumento da energia vibracional ou transições entre estados eletrônicos de energia. Para que uma transição ocorra, a energia absorvida deve ser igual ou superior à diferença entre o estado eletrônico inicial e um de maior energia. Essa diferença, constante e característica da estrutura molecular, corresponde a uma onda cujo comprimento é o comprimento de onda de excitação. Uma molécula excitada voltará ao estado inicial ou fundamental ao emitir energia na forma de calor e/ou fótons. O comprimento de onda de emissão dos fótons é também equivalente à diferença entre dois estados energéticos e é caracterísitco da estrutura molecular.

Fluorescência ocorre quando uma molécula absorve fótons do espectro de luz do UV ao visível (200-900 nm) levando à transição para estados eletrônicos de alta energia. A molécula, então, emite fótons ao retornar para o estado eletrônico fundamental em até $10^{-9}$ s. Certa quantidade de energia dentro da molécula é perdida por meio de calor ou vibrações de tal sorte que a energia emitida é menor que a energia de excitação, ou seja, o comprimento de onda de emissão é sempre maior que o de excitação. A diferença entre esses dois comprimentos de onda é chamada de 'Stokes shift'.

As proteínas contêm dois resíduos de aminoácidos aromáticos que podem contribuir para a fluorescência no UV: tirosina e triptofano. Compostos fluorescentes ou fluoróforos podem ser identificados e quantificados com base em propriedades de excitação e emissão. A magnitude da intensidade de fluorescência depende de propriedades intrínsecas do composto e de parâmetros experimentais, os quais incluem a intensidade da luz absorvida e a concentração do fluoróforo na solução. A espectroscopia de fluorescência é uma técnica muito sensível, razão pela qual solventes devem apresentar o mais alto grau de pureza possível e é essencial evitar que solventes e materiais químicos

se contaminem. Caso contrário, baixos níveis de fluorescênia podem ser mascarados por altos níveis provenientes de contaminantes (Lakowickz, 1983).

**Efeito de Temperatura, pH e Solvente na Fluorescência**

Todos os fluoróforos estão sujeitos a variações de intensidade em função da temperatura. Em geral, a intensidade da fluorescência decresce com o aumento de temperatura em razão de colisões moleculares que ocorrem mais freqüentemente a altas temperaturas. Essas colisões diminuem a energia do estado excitado que resulta na fluorescência. A resposta de um composto à variações de temperatura é única e durante a realização do experimento deve-se manter constante a temperatura.

Além da temperatura, o pH é uma variável importante. Variações de fluorescência devido a alterações de pH são causadas pelas diferentes espécies ionizáveis formadas em razão dessas alterações. Os resultados dessas variações de pH podem ser drásticos já que novas formas ionizáveis do composto são formadas.

As características do solvente usado nos experimentos de fluorescência devem ser cuidadosamente observadas. Quando um fluoróforo é transferido para um ambiente menos polar, observa-se um 'blue shift' no espectro de emissão, ou seja, há um deslocamento do comprimento de onda em direção à faixa do azul. Se ocorrer o contrário e a transferência se der para um solvente polar, o fluoróforo estará mais exposto o que se traduz por um deslocamento do comprimento de onda em direção à faixa do vermelho, o chamado 'red shift'. Esses deslocamentos sugerem alterações na estrutura tridimensional da proteína e permitem estudar o microambiente do fluoróforo.

**Atenuação da Fluorescência**

Atenuação ou 'quenching' da fluorescência refere-se a qualquer processo que resulte em redução da intensidade de fluorescência de uma substância. Na atenuação colisional ou dinâmica, o fluoróforo e atenuador entram em colisão. O atenuador deve deslocar-se até o fluoróforo enquanto este se encontra no estado excitado. Ao ocorrer o contato, o fluoróforo retorna ao estado fundamental sem emitir fóton. O outro tipo de atenuação é o estático. Assim como no dinâmico, o contato molecular entre fluoróforo e

atenuador se faz necessário. No contato, ocorre a formação de um complexo que não é fluorescente. A formação do complexo no estado fundamental resulta em perturbação do espectro de absorção do fluoróforo. O mesmo não ocorre com a atenuação dinâmica, uma vez que esta só afeta o estado excitado do fluoróforo (Eftink, 1991).

Oxigênio molecular é um dos mais bem conhecidos atenuadores colisionais capaz de atenuar a fluorescência de quase todos os fluoróforos conhecidos. Podemos citar ainda peróxido de hidrogênio, acrilamida, $I^-$, purinas e pirimidinas. Há uma variedade de atenuadores úteis em estudos de fluorescência de proteínas, especialmente no que diz respeito à acessibilidade de resíduos de triptofano e à permeabilidade da proteína ao atenuador.

Cargas elétricas afetam a atenuação da fluorescência, já que fluoróforo e atenuador precisam estar em contato próximo. Assim, um atenuador iônico de carga negativa como o $I^-$ será repelido por cargas também negativas que estejam circundando um fluoróforo. De maneira oposta, se o ambiente no qual se encontra o fluoróforo for positivamente carregado, ele será atraído. Repulsão e atração dependem da força iônica do solvente e devem decrescer em soluções de alta força iônica. Atenuadores neutros como oxigênio e acrilamida, por sua vez, não devem ser sensíveis às cargas no ambiente do fluoróforo.

O interior das proteínas é considerado não-polar assim como são não-polares os resíduos de triptofano. Desta forma, espera-se que ao menos alguns desses resíduos estejam no interior da molécula e inacessíveis ao atenuador na superfície. Para a atenuação da fluorescência dos resíduos no interior da proteína, é necessário que o atenuador possa penetrar a molécula e chegar até o fluoróforo. Conseqüentemente, a comparação de atenuação da fluorescência resultante do emprego de atenuadores carregados e não-carregados pode revelar a permeabilidade relativa da macromolécula a essas substâncias. De forma semelhante, o uso de atenuadores que não conseguem adentrar moléculas protéicas poderia ser empregado para determinação da fração da fluorescência total referente a resíduos de triptofano localizados na superfície da molécula (Sultan *et alii*, 2006).

## Desnaturação

A desnaturação de uma molécula biologicamente ativa pode ser vista como qualquer perda de estrutura que implique perda de atividade biológica. O desdobramento de uma proteína pode ser induzido por extremos de temperatura, pH, altas concentrações de agentes caotrópicos e alta pressão. O desdobramento global de uma proteína de um estado nativo ordenado (N) – com pequeno número de microestados – para um estado desordenado desdobrado (U) – com grande número de microestados – pode fornecer informações termodinâmicas importantes sobre a estabilidade de uma proteína. O principal entre esses parâmetros é a variação de energia livre N↔U que é uma medida da estabilidade do estado N em relação ao estado U. Dependendo do tipo de perturbação usada para induzir o desdobramento (temperatura, pH, desnaturante, pressão), parâmetros termodinâmicos adicionais são obtidos para descrever o processo. Os parâmetros termodinâmicos permitem, ainda, comparação de estabilidade entre proteínas e entre mutantes de uma mesma proteína bem como observação do efeito de modificações no microambiente de uma única proteína.

Para acompanhar a transição N↔U é necessário um sinal – espectroscópico, por exemplo - que seja diferente para os estados nativo e desnaturado. Geralmente usa-se CD, espectroscopia no UV-visível (absorbância) ou fluorescência. A representação N↔U é o modelo mais simples para descrever a transição conformacional de uma proteína. Uma questão relevante é determinar se o processo de desdobramento é de dois-estados ou de multi-estados, ou seja, se há ou não um ou mais intermediários no processo. No caso do modelo de dois estados, a curva de desdobramento apresenta forma sigmóide caracterísitca, há coincidência entre as curvas de transição monitoradas por diferentes técnicas e, também, equivalência entre valores de $\Delta H$ obtidos a partir do gráfico de van't Hoff e do calculado experimentalmente por microcalorimetria (Kelly & Price, 1997).

### Desnaturação Química

Nessa abordagem, a extensão do desdobramento de uma proteína é medida em diversas concentrações de um agente caotrópico, uréia e cloreto de guanidina (GuHCl) os mais usados. No modelo dois estados, as formas N e U estão em equilíbrio entre si e a análise matemática dos dados permite calcular, por exemplo, a variação da energia livre

de Gibbs (ΔG) em cada concentração. Por meio de extrapolação linear para concentração zero de desnaturante, o $\Delta G^{25}_{H2O}$ pode ser calculado e representa a energia livre da proteína no estado nativo. No caso do envolvimento de intermediários no processo de desdobramento da proteína, a análise das curvas para obtenção da estimativa das estabilidades referentes aos vários estados é mais complexa.

**Desnaturação Térmica**

Este método envolve a análise da dimensão do desdobramento da proteína em diferentes temperaturas. O exame das mudanças na constante de equilíbrio resultantes da alteração da temperatura pode ser feito utilizando-se equações termodinâmicas padrões. Valores de ΔG podem ser calculados na temperatura de interesse, por exemplo 25 °C.

O aumento da temperatura afeta, a princípio, as interações necessárias para manutenção da estrutura terciária das proteínas. Quando essas interações são rompidas, a molécula adquire uma estrutura mais flexível e ocorre a exposição de determinados grupos. Até esse estágio, a proteína geralmente consegue retornar à sua estrutura nativa se não houver aumento da temperatura. Havendo aumento, no entanto, ligações de hidrogênio responsáveis por estabilizar as hélices e as folhas ß começam a se romper, o que permite interação com a água, formação de novas ligações de hidrogênio e exposição de grupos hidrofóbicos ao solvente. Para a maioria das proteínas, essas mudanças resultam em desnaturação, já que o estado nativo da proteína não é atingido com a redução da temperatu

# OBJETIVOS

**“Advances are made by answering questions. Discoveries are made by questioning answers.”**
**Bernard Haisch**

# OBJETIVOS

**OBJETIVO GERAL:**

O objetivo geral do nosso trabalho é estudar o comportamento da POPTb em solução por meio de sua caracterização estrutural. As propriedades moleculares e funcionais dessa protease nos levam a acreditar no seu potencial como alvo para o desenvolvimento racional de drogas, tão necessárias no combate à infecção causada pelo *T. brucei*. Nossos objetivos específicos são:

- Investigar aditivos que possam ser usados em experimentos de cristalização.
- Investigar aditivos capazes de manter a atividade da enzima durante estocagem
- Caracterizar a estabilidade estrutural da enzima

# RESULTADOS

**“The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.”**
**Sir William Bragg**

# RESULTADOS

Os resultados deste estudo estão apresentados na forma de manuscritos a serem submetidos à publicação em periódicos internacionais indexados.

I - Purificação da Enzima

II - Manuscrito 1

**Structural stability of *Trypanosoma brucei* prolyl oligopeptidase**

III – Manuscrito 2

**Thermal denaturation studies of *Trypanosoma brucei* prolyl oligopeptidase.**

## PURIFICAÇÃO DA ENZIMA

*E. coli* BL21(DE3) foram transformadas com o vetor pET15b contendo o gene de 2.097 pb que codifica para a POPTb. Bactérias transformadas foram crescidas a 37 ºC sob agitação até atingirem densidade óptica $A_{600}$ = 0,6. A temperatura foi alterada para 16 ºC e a expressão da proteína induzida pela adição de 0,1 mM isopropylthio-ß-D-galactosídeo (IPTG) durante 5h. As células foram então separadas, lisadas por sonicação e centrifugadas a 16.000 x *g* por 80 minutos a 4ºC. O sobrenadante foi submetido a cromatografia de afinidade em coluna contendo Agarose-níquel e a proteína ligada foi eluída com 250 mM imidazole (Fig. 1). A proteína purificada foi dialisada contra 25 mM HEPES pH 7.5.

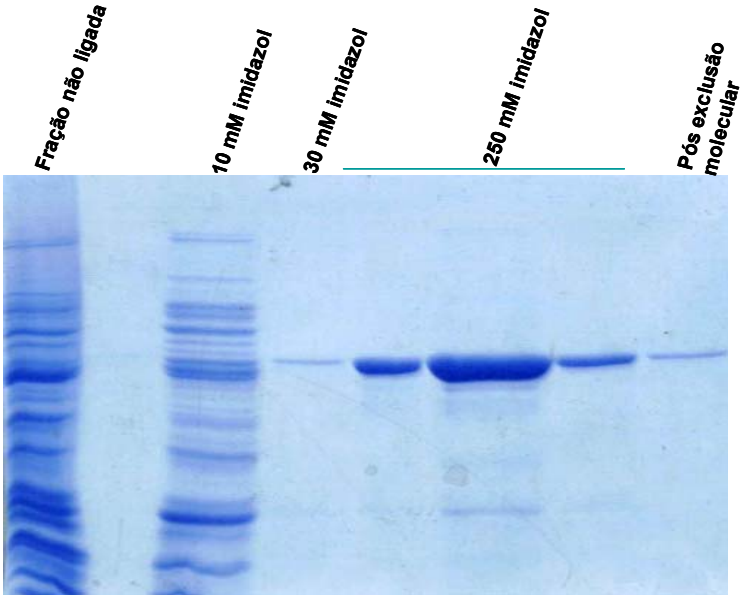


**Figura 1 Purificação da POPTb.** Extrato enzimático foi submetido à cromatografia de afinidade em Agarose-níquel. A enzima foi eluída com imidazol 250 mM, concentrada e recromatografada em coluna Superose-12. As frações ativas foram submetidas a SDS-PAGE 10% sob condições redutoras e o gel corado com Coomassie blue.

# MANUSCRITO 1

# STRUCTURAL STABILITY OF *Trypanosoma brucei* PROLYL OLIGOPEPTIDASE

Meire Maria de Lima[1], Izabela M. D. Bastos[1], David Neves[1], Francisco Javier Medrano[3], João Alexandre R. G. Barbosa[4], Philippe Grellier[5], Sônia Maria de Freitas[2]*, and Jaime Martins de Santana[1]*.

[1] Laboratório da Interação Parasito-Hospedeiro, Faculdade de Medicina, Universidade de Brasília, Brazil

[2] Laboratório de Biofísica, Instituto de Ciências Biológicas, Universidade de Brasília, Brazil

[3] Universidade Estadual de Campinas, Campinas, Brazil

[4] Centro de Biologia Molecular Estrutural, Laboratório Nacional de Luz Síncrotron, Campinas, Brazil

[5] USM 504 Muséum National d'Histoire Naturelle, Paris, France

*** Corresponding authors.** Laboratório de Interação Parasito-Hospedeiro, Faculdade de Medicina, Universidade de Brasília, 70.910-900 Brasília DF, Brazil; e-mail: jsantana@unb.br; Tel (55) 61 33072256. Laboratório de Biofísica, Instituto de Biologia, Universidade de Brasília, 70910-900, Brasília DF, Brazil; e-mail: nina@unb.br; tel (55) 61 33072192.

## Abstract

*Trypanosoma brucei* Prolyl Oligopeptidase (POPTb) is possibly involved in the pathogenesis of sleeping sickness. It is able to hydrolyze proline-containing peptide hormones and therefore considered a good drug target. As the crystal structure of POPTb has not been solved yet, we used circular dichroism and fluorescence spectroscopy to gain some knowledge on the enzyme structure, stability and on additive effects for both crystallization assays and storage. Thermodynamic and structural parameters were calculated from chemical denaturation and fluorescence quenching studies, respectively. POPTb is not a very stable protein as indicated by $\Delta G^{25}_{H2O}$ of 3.60 ± 0.27 and 3.70 ± 0.44 kcal.mol$^{-1}$ derived from guanidine hydrochloride (GuHCl) and urea unfolding assays, respectively. While cetyl trimethyl ammonium bromide (CTAB) induces structural changes on POPTb that result in nearly total loss of its activity, that induced by sorbitol results in small loss. Stern-Volmer constants show dependence on pH and indicate heterogeneity in the environment of the surface-accessible tryptophan residues. POPTb molecule seems to be less subjected to conformational changes at neutral and alkaline pH. However, at acidic pH conformational changes near tryptophan environments lead to the exposure of these residues. In this condition, POPTb decrease in activity suggests tryptophan residues may be close to the enzyme active site.

*Keywords:* Prolyl Oligopeptidase; Protein stability; Chemical denaturation; Fluorescence quenching, *Trypanosoma brucei*

# 1. Introduction

The α/β hydrolase superfamily of proteins is comprised of members which can fulfill a great diversity of catalytic and non-catalytic functions. These proteins display the so called α/β fold, characterized by a β–sheet core of five to eight strands connected by α–helices to form a α/β/α sandwich [1]. The α/β hydrolase fold family includes proteases, lipases, esterases, dehalogenases, peroxidases and epoxide hydrolases, making it one of the most versatile and widespread protein folds known [2]. A number of proteins presenting such fold have been found to be involved in diseases such as autism [3, 4] and Mast Syndrome [5].

Prolyl oligopeptidases (POP; EC 3.4.21.26) are members of the S9 serine protease family which present the α/β hydrolase fold on the catalytic domain and the β-propeller domain that forms a tight barrel-shaped lid over the active site [6]. These enzymes preferentially cleave peptide bonds on the C-terminal side of prolyl residues, show a high degree of amino acid sequence conservation and have their catalytic triad comprised of the residues Ser, Asp and His [7, 8].

POPs are ubiquitous peptidases found in many organisms including prokaryotes, yeast, higher eukaryotes and plants. On account of their activities, POPs are considered potential drug targets for the treatment of diseases such as Huntington, Parkinson and Alzheimer [9]. We have demonstrated that *Trypanosoma cruzi*, the etiological agent of Chagas` disease, secrets a prolyl oligopeptidase (POPTc80) that mediates hydrolysis of collagen [10] and plays a major role in the entry of the parasite into mammalian host cells through degradation of extra cellular matrix components as evidenced by the employment of specific inhibitors [8, 11]. *Trypanosoma brucei* prolyl oligopetidase (POPTb) may be involved in the pathogenesis of sleeping sickness since it cleaves human hormone

peptides such as thyrotropin-releasing hormone and gonadotropin-releasing hormone and, thus, contributing to hypogonadism and hypothyroidism commonly presented by untreated patients [12]. Both trypanosome orthologue peptidases show features of virulence factors and therefore are considered potential drug targets.

Although crystallographic experiments of POPTb are being carried out, no crystal structure is yet available. Given the importance of a protein full characterization with respect to its function as well as its structure, circular dichroism and fluorescence spectroscopy experiments were conducted. These methods are important tools in helping to understand how proteins achieve specific native structures in living cells [13] and to improve their stability under non-physiological conditions [14, 15], a challenge for biotechnological and pharmaceutical uses of proteins and enzymes. We were interested in gaining some insight into recombinant POPTb structure and stability in solution and into additives which could be used both for crystallization and storage of the enzyme for longer periods while keeping it active.

In this work, we describe the effects of denaturants and stabilizing agents on the structure and stability of POPTb. GuHCl is stronger a denaturant than urea and while sorbitol is able to stabilize the enzyme, CTAB drastically reduces its ability to hydrolyze the substrate. Fluorescence quenching experiments show that at pH 7.5 the molecule is less subjected to conformational changes and that some tryptophan residues are located in the vicinity of the enzyme active site. Our results demonstrate that POPTb unfolding process follows a two-state model.

## 2. Materials and methods

### *2.1. Materials*

GuHCl (G3272), CsCl (C3309), D-sorbitol (S7547), HEPES (H4034 and H8651), Trizma (T6791), CTAB (H9151) and Sodium Acetate (S9513) were purchased form Sigma Chemical Co. (St. Louis, MO). Urea (V3175) was from Promega Corporation and acrylamide from Bio-Rad Laboratories, Inc (CA, USA).

*2.2. Enzyme preparation*

The active recombinant POPTb was produced in *E. coli* BL21(DE3) and purified by affinity chromatography. These data will be published elsewhere. The purified protein was stored in 50% (v/v) glycerol at -20 ºC after dialysis against 25 mM HEPES pH 7.5. Concentration of the protein was determined experimentally using the molar absorption coefficient ε ($M^{-1}$ $cm^{-1}$) value of 91220 at 280 nm.

*2.3. Circular dichroism spectroscopy*

Circular dichroism (CD) measurements were recorded with a Jasco J-810 (Japan Spectroscopic, Tokyo) spectropolarimeter calibrated with an aqueous solution of (1R)-(-)-10-camphor-sulfonic acid (CSA). CSA CD spectrum provides convenient CD peaks, a negative CD band in the far-UV region (192.5 nm) and a positive one in the near-UV region (290.5 nm). The ratio of the absolute CD intensities at these wavelengths should be 2.05 or greater and provides an important routine check on the performance of the instrument [16]. The spectropolarimeter was equipped with a Peltier-type temperature controller and a thermostated cell holder, interfaced with a cell bath. The CD spectra were generated using a 0.1-cm pathlength quartz cuvette at a protein concentration of 0.1 mg/mL in 2.5 mM HEPES pH 7.5 in the presence or absence of urea, GuHCl, sorbitol or

CTAB. Five consecutive scans were accumulated and the average spectra values recorded. Ellipticity values ($[\theta]_{obs}$) were obtained in mdeg directly from the spectropolarimeter and converted to the mean residue ellipticity $[\theta]_{MRW,}$ in $deg.cm^2dmol^{-1}$ , using the equation:

$$[\theta]_{MRW} = \frac{[\theta]_{obs}\ MRW}{10\ C l}$$

where C is the protein concentration (mg/mL), $l$ is the path length (cm) and MRW is the average residue weight, using 111.17 for POPTb. The final ellipticities were baseline corrected by subtracting the buffer`s spectra.

The evaluation of thermodynamic parameters from the chemical denaturation curves was carried out considering a N ↔ D conversion for a two-state cooperative transition, where N represents the native and D the denatured state. The protein fraction present in the unfolded conformation ($f_U$), equilibrium constant (K) and Gibbs free energy change (ΔG) were calculated using the following equations:

$$f_U = (y_F - y) / (y_F - y_U) \quad (1)$$

$$K = [U] / [N] = f_U / (1-f_U) \quad (2)$$

$$\Delta G^0 = -RTlnK = -RTln\ [(y_F - y) / (y_F - y_U)], \quad (3)$$

where $y_F$ and $y_U$ represent the amount of $y$ in the folded and unfolded states, respectively. These data were fitted using the Origin software (Microcal Software Inc., Northampton, MA).

*2.4. Chemical denaturation*

In the isothermal unfolding experiments, 0.1 mg/mL POPTb was incubated in the presence of urea (0-8 M) or GuHCl (0-7 M), upon increments of 0.2 M, in HEPES (25 mM pH 7.5) at 25ºC for 24 h to allow time for the samples to reach equilibrium and no further spectral changes to occur. Fluorescence spectra of the enzyme were monitored with a luminescence spectrometer (Aminco Bowman Series 2 - SLM Aminco); a 1 x 1 cm pathlength cuvette with a water-jacketed cell previously stabilized at 25ºC was used, and all spectra were corrected for the buffer emission. The excitation wavelength was set at 295 nm and the fluorescence emission spectra were scanned from 310 to 420 nm by increments of 1 nm. CD measurements were recorded as described above and both thermodynamic and unfolding parameters derived from the transition curves were calculated in the same way as the fluorescence measurements.

*2.5. Fluorescence quenching experiments*

Aliquots of the concentrated stock solutions of either acrylamide or CsCl were sequentially added to 0.1 mg/mL of enzyme in 25 mM of the following buffers: sodium acetate pH 5.0, Hepes pH 7.5 and Tris-HCl pH 9.0. The fluorescence emission spectra were scanned from 310 to 400 nm by increments of 1 nm upon excitation at 295 nm. The data were corrected for the baseline contributions of the buffers with the same concentration of both quenchers and then analyzed by fitting to the Stern-Volmer equation [17]

$$Fo/F = 1 + K_{sv}[Q] \quad (4)$$

in which *Fo* and *F* are the fluorescence intensities in the absence or presence of the quencher, respectively. $K_{SV}$, calculated as the slope of the Stern-Volmer plot, is the Stern-Volmer quenching constant and a measure of the accessibility of tryptophans to acrylamide and CsCl. [Q] is the quencher concentration, considering the classical collisional quenching.

## 3. Results

### *3.1. POPTb shows instability in the presence of chemical denaturants*

Chemical denaturation and fluorescence quenching studies were performed to gain some knowledge with respect to the positioning of tryptophan residues in the molecule and to the micro-environment of such fluorophores. POPTb has 9 tryptophan residues, but no information is available as to them being buried or exposed in the molecule. GuHCl and urea denaturation of the protein were followed by both CD and fluorescence spectroscopy to monitor changes in secondary and tertiary structures of the enzyme, respectively. The fluorescence emission spectrum presents a maximum at 336 nm in the absence of denaturant agents (Fig. 1), which is a characteristic of tryptophan residues partially buried in the protein [17,18]. However, different degrees of tryptophyl residues exposure were observed upon the addition of such agents (Fig. 1). With increasing GuHCl concentrations, quenching of tryptophan intrinsic fluorescence takes place as well as an evident red shift of the emission spectra (Fig. 1A). The $\lambda_{max}$ for emission changes from 336 to 351 nm, which is a feature of tryptophan residues being totally exposed to the solvent. Upon incubation of the protein in the presence of urea, the fluorescence emission

spectrum maximum shifts less (to 345 nm) but indicates the residues are also exposed to the solvent (Fig. 1B). In both cases, the redshifts show the tryptophan residues are placed in a more hydrophilic environment as denaturation occurs. This scenario is expected upon unfolding and consequent loss of protein structure.

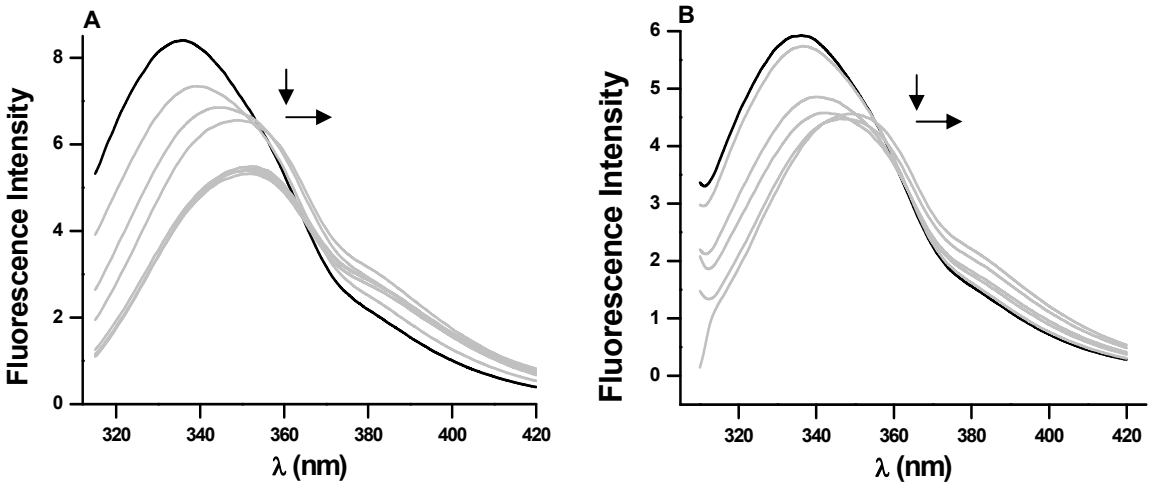


**FIGURE 1 Increasing concentrations of denaturant lead to quenching of fluorescence with redshift**. 0.1 mg/mL POPTb was incubated with various concentrations of (A) GuHCl (0-7 M) and (B) Urea (0-8 M) in 0.2 M increments in HEPES buffer (25 mM pH 7.5) at 25ºC for 24h and fluorescence emission spectra were recorded.

The unfolding of the enzyme was also investigated by recording the molar ellipticity at 222 nm. The analysis of the CD data to yield fractional denatured state as a function of denaturant concentration demonstrates that POPTb denatures faster in the presence of GuHCl than it does in that of urea (Fig. 2A). POPTb begins to unfold beyond 1.2 M and complete unfolding of the protein is achieved in 5.7 M of GuHCl as opposed to 2.7 and 7.4 M for urea. The concentration of denaturant at which half of the enzyme population is in the denatured state and the other half in the native state ($C_{1/2}$) and a

measure of the ΔG dependence on denaturant concentration (*m* values) are listed in Table 1. The concentration of urea at halfway the denaturation is slightly over 30% of the concentration of GuHCl necessary to reach the same point.

**Table 1**

Thermodynamic parameters for the chemical unfolding of POPTb obtained from far-UV CD measurements.

| ***Denaturant*** | $\Delta G_{H2O}$ (kcal.mol$^{-1}$) | $C_{1/2}$ (M) | ***m*** (kcal.mol$^{-1}$.M$^{-1}$) |
|---|---|---|---|
| Gdn-HCl | 3.60 ± 0.27 | 3.9 | 1.0 ± 0.1 |
| Urea | 3.70 ± 0.44 | 5.1 | 0.7 ± 0.1 |

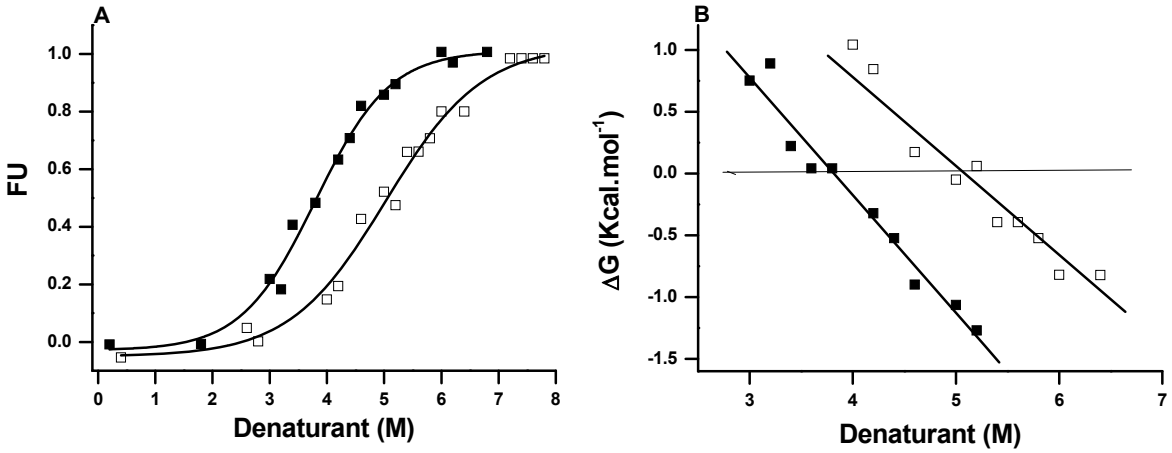


**FIGURE 2 Effect of GuHCl (■) and Urea (□) on POPTb at 25ºC.** (A) Equilibrium denaturation followed by CD**.** Molar ellipticity was recorded at 222 nm in the far-UV region and the fraction of unfolded protein plotted as a function of denaturant concentration. (B) Change of the free energy (ΔG) values as a function of denaturant concentration. ΔG values were calculated according to Eq. 3 assuming two-state unfolding processes.

Since the protein was exposed to increasing concentrations of denaturants at constant temperature, we were able to calculate, besides the protein fraction present in the

unfolded state, $f_U$ (Eq. 1), the values for the equilibrium constant of the unfolding process, $K$ (Eq. 2), and also $\Delta G^{25}$ in the absence of denaturant. By means of Eq. 3, the data from Fig. 2A were used to obtain values of $\Delta G^{25}$ at different denaturant concentrations. The plotted results show the effect of GuHCl on the protein is stronger than that of urea (Fig. 2B).The Gibbs free energy values calculated at 25 $^{o}$C in the absence of denaturant, $\Delta G^{25}_{H2O}$, corresponding to the stability of the protein, were acquired through the linear extrapolation of the data in Fig. 2B to zero denaturant concentration [19,20] and are summarized in Table 1. The low values are in agreement with data calculated for other proteins [21-23] whose structures are unstable in the same conditions. These results correlate well with our previous assays demonstrating that the recombinant enzyme is not able to keep its activity for long upon storage in buffer alone.

*3.2. Quenching of POPTb tryptophan fluorescence by acrylamide and CsCl*

Fluorescence quenching experiments by means of different quenchers make it possible to examine the microenvironment of tryptophan residues. Therefore, we investigated the effects of acrylamide and $Cs^+$ ions, a neutral and charged quencher, respectively, on POPTb tryptophan fluorescence at different pH values through the calculated Stern-Volmer quenching constants ($K_{sv}$) as in Eq. 4. The excitation wavelength was set to 295 nm to excite the tryptophan residues and to minimize excitation of the 28 tyrosine residues present in POPTb.

POPTb quenching by $Cs^+$ ions is clearly biphasic at all pHs (Fig. 3B and Table 2), indicating CsCl quenches POPTb intrinsic tryptophan fluorescence by dynamic or collisional quenching and that POPTb has different populations of tryptophan residues in different microenvironments [24]. The $K_{sv}$ calculated for the first phase of $Cs^+$ ions at pHs 5.0 and 7.5 is 1.7 and 1.9-fold higher, respectively, than that determined for free tryptophan (8.1 ± 0.4 $M^{-1}$), as currently reported [25]. At pH 9.0, however, the $K_{sv}$ is a little lower (7.38 $M^{-1}$) than that calculated for free tryptophan residues. In the second

phase, $Cs^+$ ions show little ability to quench the fluorescence at pH 7.5 ($K_{sv}$ = 0.28 $M^{-1}$) and 9.0 ($K_{sv}$ = 0.57 $M^{-1}$) and can quench it to a certain extent at pH 5.0 ($K_{sv}$ = 1.97 $M^{-1}$) (Table 2). Such values could be the result of conformational changes that take place after the initial, fast quenching of POPTb by CsCl, which vary according to the solvent pH. Chemical denaturation studies (Fig. 1) suggest tryptophyl residues are partially buried in the protein as given by $\lambda_{max}$ of 336 nm in the absence of denaturant. It corresponds to the second phase of quenching at pH 7.5 and demonstrates the study of the microenvironment of fluorophores is more accurate when allied to fluorescence quenching experiments.

Acrylamide quenches the tryptophyl fluorescence preferentially by collisional quenching. Fig. 3A and Table 2 show that only at pH 5.0 does acrylamide quench the fluorescence by the two steps. The experimental data used to calculate the Stern-Volmer constants for the first phase of quenching at the different pH values and quenchers are shown in Table 3.

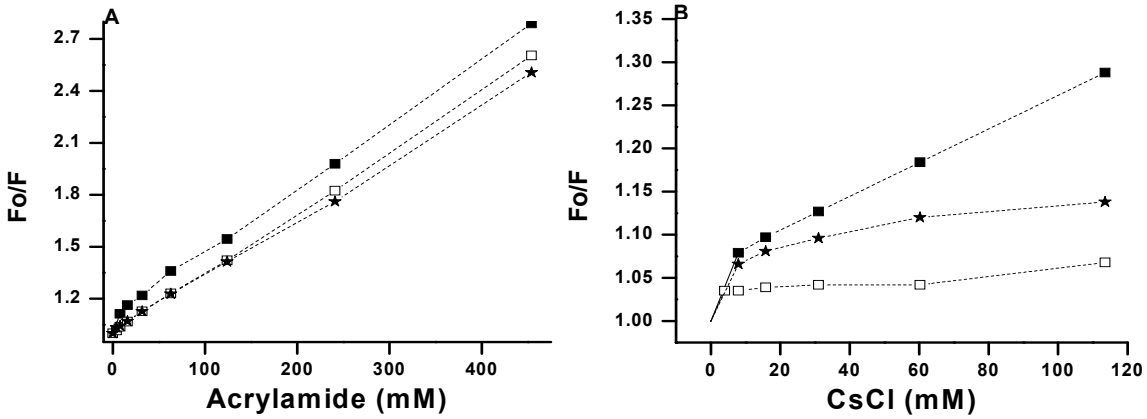


**FIGURE 3 Stern-Volmer plots of fluorescence quenching of POPTb as a function of pH by Acrylamide (A) and CsCl (B).** The studies were carried out at room temperature and fluorescence was monitored at pH 5.0 (■), pH 7.5 (□) and pH 9.0 (★).

**Table 2** Stern-Volmer constants for the fluorescence quenching of POPTb (at 25ºC) at different pH values. All SD (standard deviation) values were under 1.

| pH | Quencher | Stern Volmer Constant ($M^{-1}$) |
|---|---|---|
| | *A*crylamide[a] | 14.1 |
| | Acrylamide [b] | 3.7 |
| pH 5.0 | CsCl[a] | 13.8 |
| | CsCl[b] | 1.97 |
| | *A*crylamide | 3.50 |
| pH 7.5 | CsCl[a] | 15.0 |
| | CsCl[b] | 0.28 |
| | Acrylamide | 3.30 |
| pH 9.0 | CsCl[a] | 7.38 |
| | CsCl[b] | 0.57 |

[a] $K_{sv}$ first phase of quenching [b] $K_{sv}$ second phase of quenching

These results show, as well, that acrylamide quenching of POPTb is not significantly affected by changes in pH and that the tryptophan residues are buried the most at pH 9.0 and the least at pH 5.0. These data correlate well with our previous results showing that the activity of trypanosome POP has a strong dependence on neutral and alkaline pH [11].

**Table 3** Experimental data of Fo/F used to calculate the Stern-Volmer constants for the first phase of quenching..

| pH | CsCl (mM) | | | | Acrylamide (mM) | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 0 | 4 | 8 | 16 |
| | Fo/F | | | | Fo/F | | | |
| 5.0 | 1.00 | 1.024 | 1.042 | 1.057 | 1.00 | 1.059 | 1.113 | 1.163 |
| 7.5 | 1.00 | 1.027 | 1.030 | 1.035 | | | | |
| 9.0 | 1.00 | -- | 1.048 | 1.058 | | | | |

*3.3. Fluorescence experiments show CTAB and sorbitol have opposing effects on POPTb*

A concern in our laboratory has been the storage of proteins in concentrated solutions in such a way that enzyme activity is preserved. Lyophilization was unsuccessfully tried, since we were unable to regenerate active material. We have been using glycerol at 50% (v/v) as a protective agent, which allows the solution to be stored at -20 ºC without freezing. On the other hand, in this concentration such solutions are too viscous for other procedures such as chromatography [26] and crystallization assays. Since no crystallization condition for solving the three-dimensional structure of this protein has been found and it is well known that polyols [27-29] as well as detergents [15] enhance the stability of enzymes, we addressed experiments with the stabilizing agents CTAB and sorbitol which could provide information on the best conditions for both storing and improving the stability of this protein prior to crystallization screening assays. CTAB and sorbitol have different effects on POPTb structure, as shown in Fig. 4. CTAB causes an alteration on the tertiary structure of the protein (Fig. 4A), suggested by the spectra redshift from 335.8 to 341.4 (inset Fig. 4A). Besides these changes, the increase in surfactant concentration also leads to higher fluorescence emission. Sorbitol, on the other hand, causes no shift (Fig. 4B) and induces preferential hydration of the protein leading to quenching of fluorescence, as shown in the inset of Fig. 4B.

We also tested these stabilizing agents for their ability to maintain the level of POPTb activity. The addition of CTAB to a final concentration of 0.1 mM drastically reduces enzyme activity. Its ability to hydrolyze the fluorogenic substrate N-Suc-Gly-Pro-Leu-Gly-Pro-AMC drops to less than 1%. In contrast, sorbitol does not affect enzyme activity as much since in the presence of 0.5 M sorbitol the enzyme retains nearly 80% of its activity (data not shown). Far-UV CD experiments demonstrate how much these agents affect the enzyme secondary structure (Fig. 5). The addition of 0.1 or 0.5 mM CTAB, although enough to almost halt enzyme activity, has no significant effect on POPTb structure. Higher concentrations, however, lead to increase in signal and hence a more packed-in conformation (Fig. 5A). The addition of sorbitol does not result in change of secondary structure up to the concentrations used (Fig. 5B).

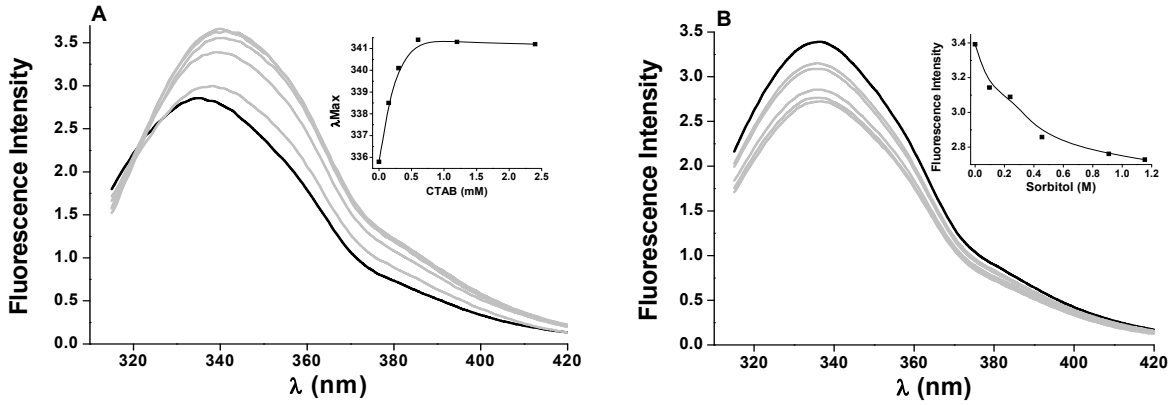


**FIGURE 4 Stabilizing agents have opposing effects on POPTb structure.** Fluorescence spectra of POPTb in 25 mM HEPES buffer pH 7.5 in the absence or presence of increasing concentrations of (A) CT*A*B (0-2.4 mM) and (B) Sorbitol (0-1.2 M). Excitation was at 295 nm.

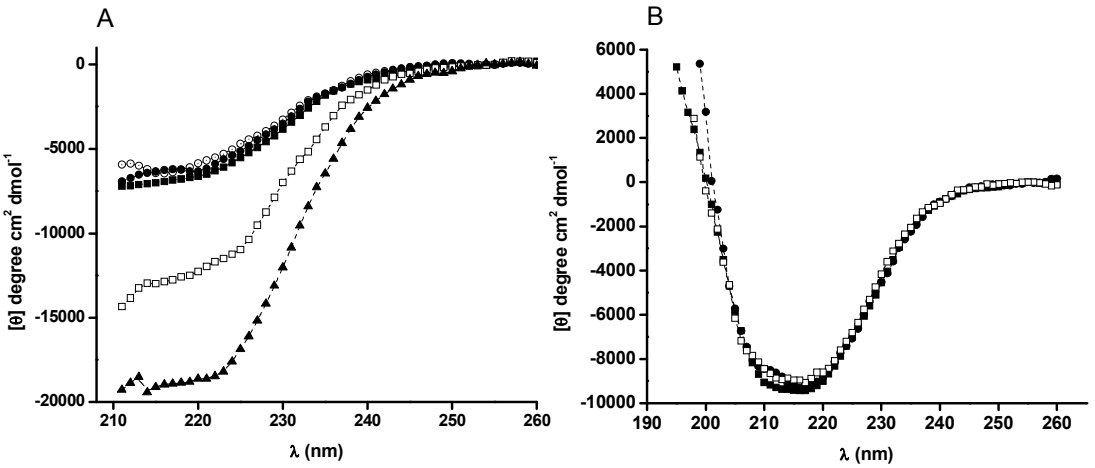


**FIGURE 5 Far-UV CD spectra of POPTb at 25ºC in the absence (■) or presence of CTAB (A) or Sorbitol (B).** CTAB was assayed at 0.1(**O**), 0.5(●), 1(□) and 5 mM(▲) and sorbitol at 0.5 (●) and 1 M(□). The experiments were carried out in 2.5 mM Hepes pH 7.5.

## 4. Discussion

Protein stability studies are commonly performed from unfolding assays in the presence of denaturants such as GuHCl and urea. GuHCl is one of the most widely used agents to denature proteins reversibly and to determine the difference in ΔG between the unfolded and the folded states [30]. We followed the equilibrium urea and GuHCl denaturations of POPTb by both CD and fluorescence spectroscopy. The addition of increasing concentrations of either denaturant to a solution of POPTb led to quenching of fluorescence emission and increase in the emission spectra maxima indicating protein

denaturation. GuHCl is very versatile as it can cause different effects on proteins according to the concentration used. It can act as a strong denaturant which can coat the exterior of proteins [30-33], assist in correct refolding [34] and stabilize proteins [35]. In the case of POPTb, it is clear that the protein stability decreases as GuHCl concentration is increased and from the values of $\Delta G^{25}_{H20}$ that POPTb is not a stable enzyme, in agreement with values obtained for unstable proteins [21-23,36]. Considering the fact that the unfolding curves have the same shape, we assumed the process of POPTb denaturation is probably cooperative, an essential step in evaluating the equilibrium thermodynamics of protein denaturation. Such a process requires that at equilibrium there are only two states (N and/or D) with no substantive populations of partially folded states during the melting of the protein [23].

As seen for other proteins, urea is a generally less potent denaturant compared to GuHCl [23,36-38]. Our results show that GuHCl is more efficient than urea in unfolding POPTb, possibly due to its natural properties of being not only a denaturant but also a strong salt. The solvent ionic strength caused by GuHCl lead to a higher denaturing effect as shown by Ferreon and Boleon (2004) for β1, RNAse A and β-lactoglobulin. However, the increase in the solvent ionic strength by the addition of NaCl to aqueous solutions does not result in an enhanced denaturing process by urea, which implies that GuHCl and urea most probably unfold proteins by means of a different molecular mechanism [38]. Granata et al. (2004) stated that GuHCl may act either by an indirect mechanism related to the modifications caused by the addition of the denaturant to the properties of water or by a direct mechanism related to the occurrence of direct favorable interactions between the polypeptide chain and the guanidinium ions.

CTAB, a cationic surfactant, was tested as for its ability to stabilize the protein, since it is known to provide an effective confinement of proteins by micellization [13].

Enzymatic assays of POPTb carried out in the presence of CTAB demonstrate almost total loss of activity even at the lowest concentration used (data not shown). We employed concentrations below, at and above the critical micelle concentration (CMC), namely 1 mM for CTAB [15]. CD assays demonstrate the dichroic signal is basically unaltered at concentrations below the CMC, whereas with increasing concentrations of the surfactant the dichroic signal increases significantly, suggesting the formation of more compact secondary structure of the protein. From 0.1 mM on, CTAB is able to abolish enzyme activity, but it only affects the structure of POPTb when the amount of surfactant is big enough for the formation of micelles (as of 1 mM). It is clear from our data that CTAB has a destabilizing effect on the enzyme much before its secondary structure is changed.

Sorbitol is a low-molecular-mass solute produced by some freeze-resistant eukaryotes during water stress and also by some heat-resistant organisms [39]. Many studies demonstrate that this component is also capable of protecting proteins against thermal inactivation [27,29,40-42] and that the overall influence of sorbitol was related with water activity or the preferential solvent interaction in the microenvironment of the enzyme [40]. In our experiments, sorbitol acted as a fluorescence quencher, but did not alter the enzyme tertiary structure. Far-UV CD spectra of POPTb in the presence of sorbitol suggest no alterations on the protein secondary structure took place either. These results can as well account for the small loss of activity (about 20%) observed in the presence of 0.5 M sorbitol (data not shown) and are in agreement with the ones reported by De Diego et al. (2004) on a stabilizing effect of sorbitol on α-chymotrypsin, a serine protease as well. Enzyme activity was preserved and they suggest the role of sorbitol is probably due to immobilization of the water medium, rather than any increase in the rigidity of the protein structure. Indeed, since sorbitol is a polyol, efficient hydrogen bonding interactions with the proteins are expected to take place [29].

Conformational changes can have a direct influence on solvent accessibility of tryptophan residues in a protein. Fluorescence quenching assays have been used as a tool to sense such exposure of fluorophores in molecules [43]. To gain information on the microenvironment of POPTb tryptophyl residues, we used acrylamide, a non charged compound known to penetrate the protein molecule [43,44] and CsCl, a charged compound known to quench the fluorescence emission of tryptophan residues mainly by collisional effects [45-48]. Stern-Volmer plots for the quenching of proteins with two different populations of tryptophan residues generally show a downward curvature characterizing two distinct steps (biphasic quenching). This feature indicates that one of the tryptophan residues population is accessible and the other is inaccessible or only slightly accessible to the quencher. In this case, the expected Stern-Volmer constant for the buried fraction of the total fluorescence is lower than that for the accessible fraction [24]. As seen for POPTb, acrylamide yields biphasic quenching patterns at acidic but not at neutral or alkaline pHs. CsCl, on the other hand, produces biphasic quenching patterns regardless of the pH values, indicating heterogeneity in the environment of the tryptophan residues.

The values for the first phase quenching by $Cs^+$ ions at acidic and neutral pHs are higher than that of free tryptophans. Since there are no obstacles for free tryptophans to access the quencher, higher values seem to be related to the existence of an unknown interaction between quencher and fluorophore that somehow increases the fluorescence quenching to levels above the one considered the highest for simple collisions [25].

The second phase, on the other hand, shows low $K_{sv}$ values due to dynamic collisional quenching and is probably associated to a buried and different tryptophan population. Quenching of POPTb by $Cs^+$ ions at acidic and neutral pHs are similar. A comparison of the results at acidic and alkaline pHs indicate that it is harder for the

quencher to reach the tryptophyl residues at the latter, which leads to a smaller quenching, and is compatible with tryptophan residues less exposed than at an acidic pH. Considering the values of *K*sv, the tryptophan residues are more exposed at neutral than at alkaline pH, most probably owing to changes at the ionic state, characteristic of solvent charges. Once simple collisions take place and tryptophan residues interact with $Cs^+$ ions, though, changes in the molecule arrangement lead to alterations in the residues environment such that the quenching in the second phase is low at neutral pH. As a consequence, the tryptophans of POPTb are essentially unquenched by CsCl in this phase. At the alkaline pH, they are slightly more quenched at this stage. At the acidic pH, the $K_{sv}$ is close to 2 indicating that the residual charge of the protein at this pH results in conformational changes which do not conceal the tryptophan residues as much. Indeed, these results support an exposed rather than a buried environment for most of POPTb tryptophans, which is in agreement with our findings that the $\lambda_{max}$ of the fluorescence emission spectrum of POPTb in the absence of denaturant is 336 nm, characteristic of partially buried residues. Taken all the results into consideration, POPTb molecule seems to be less subjected to conformational changes at neutral pH as a result of the residual charges the protein presents at this pH.

When acrylamide is used as a quencher, the same biphasic process is only seen at an acidic pH. The calculated $K_{sv}$ values increase from the alkaline to the acidic pH, indicative of residues more exposed in the latter. Since they are all close to half of the value determined for free tryptophans, collisional quenching is expected to have taken place. Stern-Volmer plots and $K_{sv}$ values show acrylamide quenching of POPTb is not significantly affected by changes in pH. The data obtained from fluorescence quenching experiments are in agreement with the pattern expected from dynamic fluorescence quenching of proteins with more than one tryptophan residue in different accessible

environments [47]. Moreover, since at an acidic pH tryptophan residues are more exposed and the enzyme is less active, the data also may suggest the proximity of at least one of these residues population to the enzyme active site.

In this study, the effects of denaturants and stabilizing agents on the recombinant prolyl oligopeptidase of *Trypanosoma brucei* were examined. GuHCl is stronger a denaturant than urea and while sorbitol is able to stabilize the enzyme, CTAB drastically reduces the enzyme ability to hydrolyze the substrate. Fluorescence quenching experiments show that at pH 7.5 the molecule is less subjected to conformational changes.

**Acknowledgements**

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico CNPq, Fundação de Amparo à Pesquisa do Distrito Federal FAPDF, Financiadora de Estudos e Projetos FINEP, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior CAPES and Associação Brasileira de Tecnologia de Luz Síncrotron ABTLuS. MML and IMDB are fellows of CNPq and CAPES, respectively

## 5. References

[1] T. Hotelier, R. Ludovic, X. Cousin, V. Negre, P. Marchot, A. Chatonnet, ESTHER, the database of the α/β-hydrolase fold superfamily of proteins, Nucleic Acids Res. 32 (2004) D145-D146.

[2] M. Nardini, B.W. Djkstra, α/β-hydrolase fold enzymes: the family keeps growing, Curr. Opin. Struct. Biol. 9 (1999) 732-737.

[3] J. Gauthier, A. Bonnel, J. St-Onge, L. Karemera, S. Laurent, L. Mottron, E. Fombonne, R. Joober, G.A. Rouleau, NLGN3/NLGN4 gene mutations are not responsible for autism in the Quebec population, Am. J. Med. Genet. B Neuropsychiatr. Genet. 132 **(**2005) 74-75.

[4] T. Ylisaukko-oja, K. Rehnstrom, M. Auranen, R. Vanhala, R. Alen, E. Kempas, P. Ellonen, J. A. Turunen, I. Makkonen, R. Riikonen, T. Nieminen-von Wendt, L. von Wendt, L. Peltonen, I. Jarvela, Analysis of four neuroligin genes as candidates for autism, Eur. J. Hum. Genet. 13 **(**2005) 1285-1290.

[5] M.A. Simpson, H. Cross, C. Proukakis, A. Pryde, R. Hershberger, A. Chatonnet, M.A. Patton, A.H. Crosby, Maspardin is mutated in mast syndrome, a complicated form of hereditary spastic paraplegia associated with dementia, Am. J. Hum. Genet. 73 (2003) 1147-1151.

[6] V. Fülop, Z. Böcskei, L. Polgár, Prolyl oligopeptidase: an anusual β–propeller domain regulates proteolysis, Cell 94 (1998) 161-170.

[7] I. Brandt, K. Vriendt, B. Devreese, J. Beeumen, W. Dongen, K. Augustyns, I. Meester, S. Scharpé, A.M. Lambeir, Search for substrates for prolyl oligopeptidase in porcine brain, Peptides 12 (2005) 2536-2546.

[8] I.M.D. Bastos, P. Grellier, N.F. Martins, G. Cadavid-Restrepo, M.R., De Souza-Ault, K. Augustyns, A.R.L. Teixeira, J. Schrével, B. Maigret, J.F. Da Silveira, J.M. Santana, Molecular, functional and structural properties of the prolyl oligopeptidase of *Trypanosoma cruzi* (POP Tc80), which is required for parasite entry into mammalian cells, Biochem. J. 388 (2005) 29-38.

[9] P.T. Männisto, J. Venäläinem, A. Jalkanen, J.A. García-Horsman, Prolyl oligopeptidase: a potential target for the treatment of cognitive disorders, Drug News Perspect. 20 (2007) 293-305.

[10] J.M. Santana, P. Grellier, J. Schrével, A.R.L. Teixeira, A *Trypanosoma cruzi*-secreted 80 kDa proteinase with specificity for human collagen types I and IV, Biochem. J. 324 (1997) 129-137.

[11] P. Grellier, S. Vendeville, R. Joyeau, I.M.D. Bastos, H. Drobecq, F. Frappier, A.R.L. Teixeira, J. Schrével, E. Davioud-Charvet, S. Sergheraert, J.M. Santana, *Trypanosoma cruzi* prolyl oligopeptidase Tc80 is involved in nonphagocytic mammalian cell invasion by trypomastigotes, J. Biol. Chem. 276 (2001) 47078-47086.

[12] M. Reincke, W. Arlt, C. Heppner, F. Petzke, G.P Chrousos, B. Allolio, Neuroendocrine dysfunction in African trypanosomiasis. The role of cytokines, Ann. N. Y. Acad. Sci. 840(1998) 809-21.

[13] D. Lu, Z. Liu, J. Wu, Structural transitions of confined model proteins: molecular dynamics simulation and experimental validation, Biophys. J. 90 (2006) 3224-3238.

[14] R. Wimmer, M. Olsson, M.T. Petersen, R. Hatti-Kaul, S.B. Petersen, N. Muller, Towards a molecular level understanding of protein stabilization: the interaction between lysozyme and sorbitol, J. Biotechnol. 55 (1997) 85-100.

[15] J. Mansfeld, R. Ulbrich-Hofmann, The stability of engineered thermostable neutral proteases from *Bacillus stearothermophilus* in organic solvents and detergents, Biotechnol. Bioeng. 97 (2007) 672-679.

[16] S.M. Kelly, T. J. Jess, N.C. Price, How to study proteins by circular dichroism, Biochim. Biophys. Acta 1751 (2005) 119-139.

[17] J.R. Lakowicz, Topics in Fluorescence Spectroscopy, Vol. 2. J.R. Lakowics, editor. Plenum Press, New York, 1991 pp. 239-242.

[18] E.A. Burstein, N.S. Vedenkina, M.N. Ivkova, Fluorescence and the localization of tryptophan residues in protein molecules, Photochem. Photobiol. 18 (1973) 263-279.

[19] R.F. Greene Jr, C.N. Pace, Urea and Guanidine Hydrochloride Denaturation of Ribonuclease, Lysozyme, α-Zhymotrypsin, and β-Lactoglobulin, J. Biol. Chem. 429 (1974) 5388-5393.

[20] S.M. Kelly, N.C. Price, The application of circular dichroism to studies of protein folding and unfolding, Biochim. Biophys. Acta 1338 (1997) 161-185.

[21] B. Vallée, C. Teyssier, R. Maget-Dana, J. Ramstein, N. Bureaud, F. Schoentgen, Stability and physicochemical properties of the bovine brain phosphatidylethanolamine-binding protein, Eur. J. Biochem. 266 (1999) 40-52.

[22] D.W. Bolen, M. Yang, Effects of guanidine hydrochloride on the proton inventory of proteins: implications on interpretations of protein stability, Biochemistry 39 (2000) 15208-15216.

[23] A.C.M. Ferreon, D.W. Bolen, Thermodynamics of denaturant-induced unfolding of a protein that exhibits variable two-state denaturation, Biochemistry 43 (2004) 13357-13369.

[24] J.R. Lakowicz, Quenching of Fluorescence, in: Principles of Fluorescence Spectroscopy, second edition, Plenum Press, New York, 1999, Chapter 8, pp. 237-265.

[25] M. Möller, A. Denicola, Protein tryptophan accessibility studied by fluorescence quenching, Biochem. Mol. Biol. Educ. 30 (2002) 175-178.

[26] N.C. Price, Conformational issues in the characterization of proteins, Biotechnol. Appl. Biochem. 31 (2000) 29-40.

[27] J.M. Obón, A. Manjón, J.L. Iborra, Comparative thermostability of glucose dehydrogenase from *Haloferax mediterranei*. Effects of salts and polyols, Enzyme Microb. Technol. 19 (1996) 352-360.

[28] R. Di Domenico, R. Lavecchia, Thermal stability of human haemoglobin in the presence of sarcosine and sorbitol, Biotechnol. Lett. 22 (2000) 335-339.

[29] L.L. Chang, D. Shepherd, J. Sun, X. C. Tang, M.J. Pikal, Effect of sorbitol and residual moisture on the stability of lyophilized antibodies: Implications for the mechanism of protein stabilization in the solid state, J. Pharm. Sci. 94 (2005) 1445-1455.

[30] R. Varhac, M. Antalík, M. Bánó, Effect of temperature and guanidine hydrochloride on ferrocytochrome c at neutral pH, J. Biol. Chem. 9 (2004) 12-22.

[31] V. Granata, P. Del Vecchio, G. Barone, E. Shehi, P. Fusi, P. Tortora, G. Graziano, Guanidine-induced unfolding of the Sso7d protein from the hyperthermophilic archaeon *Sulfolobus solfataricus*, Int. J. Biol. Macromol. 34 (2004) 195-201.

[32] A. Mukaiyama, K. Takano, M. Haruki, M. Morikawa, S. Kanaya, Kinetically robust monomeric protein from a hyperthermophile, Biochemistry 43 (2004) 13859-13866.

[33] S. Biswas, A.M. Kayastha, Unfolding and refolding of leucoagglutinin (PHA-L), an oligomeric lectin from kidney beans (*Phaseolus vulgaris*), Biochim. Biophys. Acta 1674 (2004) 40-49.

[34] B.A. Vernaglia, J. Huang, E.D. Clark, Guanidine hydrochloride can induce amyloid fibril formation from hen egg-white lysozyme, Biomacromolecules 5 (2004) 1362-1370.

[35] R. Kumar, N.P. Prabhu, M. Yadaiah, A.K. Bhuyan, Proteins stiffening and entropic stabilization in the subdenaturing limit of guanidine hydrochloride, Biophys. J. 87 (2004) 2656-2662.

[36] C.N. Pace, Determination and analysis of urea and guanidine hydrochloride denaturation curves. In: Methods in Enzymology. Enzyme Structure. Part L, Academic Press, New York, pp 266-280.

[37] G. Colonna, S.S. Alexander Jr, K.M. Yamada, I. Pastan, H. Edelhoch, The stability of cell surface protein to surfactants and denaturants, J. Biol. Chem. 253 (1978) 7787-7790.

[38] P. Del Vechio, G. Graziano, V. Granata, T. Farias, G. Barone, L. Mandrich, M. Rossi, G. Manco, Denaturant-induced unfolding of the Acetyl-esterase from *Escherichia coli*, Biochemistry 43 (2004) 14637-14643.

[39] P.H. Yancey, M.E. Clark, S.C. Hand, R.D. Bowlus, G.N. Somero, Living with water stress: evolution of osmolyte systems, Science 217 (1982) 1214-1222.

[40] G. Xie, S.N. Timasheff, Mechanism of the stabilization of ribonuclease A by sorbitol: Preferential hydration is greater for the denatured than for the native protein, Protein Sci. 6 (1997) 211-221.

[41] F. Cioci, R. Lavecchia, Sorbitol-mediated stabilization of human IgG against thermal inactivation, Biotechnol. Tech. 12 (1998) 855-858.

[42] T. De Diego, P. Lozano, S. Gmouh, M. Vaultier, J.L. Iborra, Fluorescence and CD spectroscopic analysis of the α-chymotrypsin stabilization by the ionic liquid, 1-ethyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]amide, Biotechnol. Bioeng. 88 (2004) 916-924.

[43] M.R. Eftink, C.A. Ghiron, Exposure of tryptophanil residues and protein dynamics, Biochemistry 16 (1977) 5546-5551.

[44] M.R. Eftink, C.A. Ghiron, Exposure of tryptophanil residues in proteins. Quantitative determination by fluorescence quenching studies, Biochemistry 15 (1976) 672-680.

[45] J. Schültz, P. Angelova-Dolashka, R. Abrashev, P. Nicolov, W. Voelter, Isolation and spectroscopic characterization of the structural subunits of keyhole limpet hemocyanin, Biochim. Biophys. Acta 1546 (2001) 325-336.

[46] M. Balsera, J.B. Arellano, F. Pazos, D. Devos, A. Valencia, J. De Las Rivas, The single tryptophan of the PsbQ protein of photosystem II is at the end of a 4-α-helical bundle domain, Eur. J. Biochem. 270 (2003) 3916-3927.

[47] N.A.M. Sultan, R.N. Rao, S.K. Nadimpalli, M.J. Swamy, Tryptophan environment, secondary structure and thermal unfolding of the galactose-specific seed lectin from *Dolichos lablab*: Fluorescence and circular dichroism spectroscopic studies, Biochim. Biophys. Acta, 1760 (2006) 1001-1008.

[48] K. Idakieva, N.I. Siddiqui, K. Parvanova, P. Nikolow, C. Gielenes, Fluorescence properties and conformational stability of the β–hemocyanin of *Helix pomatia*, Biochim. Biophys. Acta 176 (2006) 807-814.

# MANUSCRITO 2

# THERMAL DENATURATION STUDIES OF *Trypanosoma brucei* PROLYL OLIGOPEPTIDASE

Meire Maria de Lima[1], Izabela M. D. Bastos[1], David Neves[1], Francisco Javier Medrano[2], João Alexandre R. G. Barbosa[3], Philippe Grellier[4], Sônia Maria de Freitas[5]*, and Jaime Martins Santana[1]*.

[1] Laboratório de Interação Parasito-Hospedeiro, Faculdade de Medicina, Universidade de Brasília, Brazil

[2] Universidade Estadual de Campinas, Campinas, Brazil

[3] Centro de Biologia Molecular Estrutural, Laboratório Nacional de Luz Síncrotron, Campinas, Brazil

[4] USM 504 Muséum National d'Histoire Naturelle, Paris, France

[5] Laboratório de Biofísica, Instituto de Ciências Biológicas, Universidade de Brasília, Brazil

***Corresponding authors.** Laboratório de Interação Parasito-Hospedeiro, Faculdade de Medicina, Universidade de Brasília, 70.910-900 Brasília DF, Brazil; e-mail: jsantana@unb.br; phone (55) 61 33072256. Laboratório de Biofísica, Instituto de Biologia, Universidade de Brasília, 70.910-900, Brasília DF, Brazil; e-mail: nina@unb.br; phone (55) 61 33072192.

## Abstract

*Trypanosoma brucei*, the aetiological agent of sleeping sickness, secrets a prolyl oligopeptidase (POPTb) which is believed to be involved in the pathogenesis of this disease. It is a post-proline cleaving enzyme that hydrolyzes human hormone peptides and a good target for rational drug design. The thermal denaturation process of POPTb in the presence and absence of additives was followed by circular dichroism spectroscopy. The thermodynamic parameters were calculated and showed that whereas the effect of sorbitol is pH-independent, that of ditiotreitol (DTT) is not. Sorbitol increases enzyme stability and 0.1 mM cetyl trimethyl ammonium bromide (CTAB) decreases it. DTT, however, has the former effect in alkaline pH and the latter effect in neutral pH. The increase in temperature lowers CTAB cmc (critical micelle concentration). Since POPTb was not fully unfolded under the conditions tested and there is a divergence in the ΔG values derived from chemical and thermal unfolding experimentes, its structural stability might be higher than previously expected and intermediates might be formed in the process.

*Keywords:* Prolyl Oligopeptidase; Protein stability; Thermal denaturation; *Trypanosoma brucei*

## 1. Introduction

Sleeping sickness, also known as Human African trypanosomiasis, is caused by *Trypanosoma brucei* in 36 countries in sub-Saharan Africa. Humans become infected when they are bit by tsé-tsé flies (*Glossina* spp.) harboring the parasites. Death is certain in untreated patients and terminal stages feature neurological signs including seizures, nighttime insomnia, daytime drowsiness and coma [1]. *T. brucei* secrets a prolyl oligopeptidase (POPTb) which is thought to be implicated in the pathogenesis of sleeping sickness (our unpublished observations). Through the cleavage of human hormone peptides, the enzyme might contribute to hypogonadism and hypothyroidism commonly presented by untreated patients [2].

Members of the S9 family of serine proteases, prolyl oligopeptidases (POPs; EC 3.4.21.26) present the α/β hydrolase fold and the β-propeller domain. The β-propeller would avoid access of peptidic substrates longer than 30 amino acid residues to the active site of the enzyme localized in the interface of the domains [3]. It provides what has been considered the most distinguishing feature of these enzymes, namely the specificity for small substrates. Conversely, we have shown that *Trypanosoma cruzi*, the aetiological agent of Chagas' disease, and *T. brucei* secret POPTc80 and POPTb, respectively, which mediate hydrolysis of the much larger proteins collagen and fibronectin. The use of specific inhibitors has shown POPTc80 holds a role in *T. cruzi* entry into mammalian cells [4,5].

These enzymes, unlike most peptidases, are able to cleave peptide bonds on the C-terminal of prolyl residues and to digest small peptide-like hormones, neuroactive peptides and various cellular factors. This ability suggests a physiological role in modulating the levels of all neuronal peptides and hormones containing this residue [6].

Since a parasitic drug target is a protein that plays an essential role in the pathogen and shows distinguishing features from homologues in the host, both POPTc80 and POPTb are considered good drug-design candidates.

Limited stability under non-physiological conditions represents nowadays a bottleneck for industrial uses of enzymes. Clarifying the mechanism involved in protein denaturation is a crucial step for understanding protein stability and unfolding pathway. The aim of this work was to study the thermal denaturation process of POPTb in the presence and absence of additives by means of circular dichroism spectroscopy. The thermodynamic parameters were calculated and showed that whereas the effects of sorbitol and cetyl trimethyl ammonium bromide (CTAB) are pH-independent, that of ditiotreitol (DTT) is not. Sorbitol increases enzyme stability and CTAB decreases it. DTT, however, has the former effect in alkaline pH and the latter effect in neutral pH. POPTb was not fully unfolded under the conditions tested indicating its structural stability might be higher than previously expected.

## 2. Materials and methods

*2.1 Chemicals.* D-sorbitol, HEPES, Trizma, CTAB, Sodium Acetate and DTT were purchased from Sigma Chemical Co.

*2.2 Enzyme preparation. E. coli* BL21(DE3) was transformed with the vector pET15b containing the 2,097 bp gene coding for POPTb. The transformed bacteria were grown at 37 ºC to an optical density of $A_{600}$ = 0.6, when the temperature was shifted to 16 ºC. Induction was carried out for 5 h by the addition of 0.1 mM isopropylthio-ß-D-galactoside (IPTG). Cells were then harvested, lysed by sonication and the supernatant submitted to affinity chromatography on a nickel-Agarose resin (Novagen). Bound

rPOPTb was eluted with 250 mM imidazole and the purified protein was stored in 50 % glycerol (v/v) at -20 ºC after dialysis against 25 mM HEPES pH 7.5. The molar absorption coefficient ε ($M^{-1}$ $cm^{-1}$) value of 91220 at 280 nm was used for determination of protein concentration.

*2.3 Circular dichroism spectroscopy.* Circular dichroism (CD) measurements were recorded using a 0.1-cm pathlength quartz cuvette containing 0.1 mg/mL protein in 2.5 mM sodium acetate pH 5.0, sodium cacodylate pH 6.4, HEPES pH 7.5 and Tris-HCl pH 9.0 in the presence or absence of sorbitol, cetyl trimethyl ammonium bromide (CTAB) or DTT. The assays were carried out in a Jasco J-810 (Japan Spectroscopic, Tokyo) spectropolarimeter calibrated with (1R)-(-)-10-camphor-sulfonic acid [7] equipped with a Peltier-type temperature controller and a thermostated cell holder, interfaced with a cell bath. Thermal denaturation was monitored by far-UV CD measurements by increasing the temperature from 10 to 95 ºC, allowing temperature equilibration for 5 min before five consecutive scans were accumulated and the average spectra values recorded. Ellipticity values ($[\theta]_{obs}$) were obtained in mdeg directly from the spectropolarimeter and converted to the mean residue ellipticity $[\theta]_{MRW}$, in deg.$cm^2$$dmol^{-1}$, using the equation:

$$[\theta]_{MRW} = \frac{[\theta]_{obs}\, MRW}{10\, C l} \qquad (1)$$

where C is the protein concentration (mg/mL), *l* is the path length (cm) and MRW is the average residue weight, 111.17 for POPTb. The final ellipticities were baseline corrected by subtracting the buffers` spectra in the absence or presence of the additives.

*2.4 Thermodynamic parameters.* The evaluation of thermodynamic parameters from the thermal denaturation curves was carried out considering a N ↔ D conversion for a two-state cooperative transition, where N represents the native and D the denatured

state. The protein fraction present in the unfolded conformation ($f_U$) and equilibrium constant ($K_{eq}$) were calculated using the following equations:

$$f_U = (y_F - y) / (y_F - y_U), \quad (2)$$

$$K_{eq} = [U] / [N] = f_U / (1-f_U), \quad (3)$$

where $y_F$ and $y_U$ represent the amount of y in the folded and unfolded states, respectively. The standard enthalpy ($\Delta Hm$) and the standard entropy ($\Delta Sm$) are the slope and the intercept, respectively, from the fitted regression of $RlnK_{eq}$ versus 1/T using the van't Hoff equation:

$$LnK_{eq} = (\Delta S/R) - (\Delta Hm/R)\,(1/T), \quad (4)$$

where R is the gas constant (1.987 $cal.mol^{-1}K^{-1}$). Changes in the Gibbs free energy ($\Delta G^{25}$) were calculated from:

$$\Delta G = \Delta Hm - T\,\Delta Sm, \quad (5)$$

where T is the temperature in Kelvin (K). These data were fitted using the Origin software (Microcal Software Inc.)

## 3. Results

*3.1 POPTb partially unfolds upon heating*

The pH-dependence of POPTb thermal denaturation was analyzed by circular dichroism experiments in the far-UV region. Data thus acquired enables changes in the secondary structure to be studied. As can be seen in figure 1, the dichroic signal is reduced upon heating regardless of the pH. DTT is frequently used to reduce the disulfide bonds of proteins [8-10] and is reported to enhance the activity of enzymes [11]. Since it was previously demonstrated in our laboratory that DTT increases the activity of *T. cruzi* POP [5], we conducted the experiments in the presence or absence of DTT at pH 7.5 and 9.0. Figure 1 shows a similar pattern for the loss of protein secondary structure in the presence of DTT. The dichroic signal decreases as a function of temperature as the protein gradually unfolds. Figure 1 also shows that, under the conditions of our experiments, the denaturation of the protein was not complete as evidenced by the dichroic signal around -4000 $deg.cm^2dmol^{-1}$ in the higher temperatures.

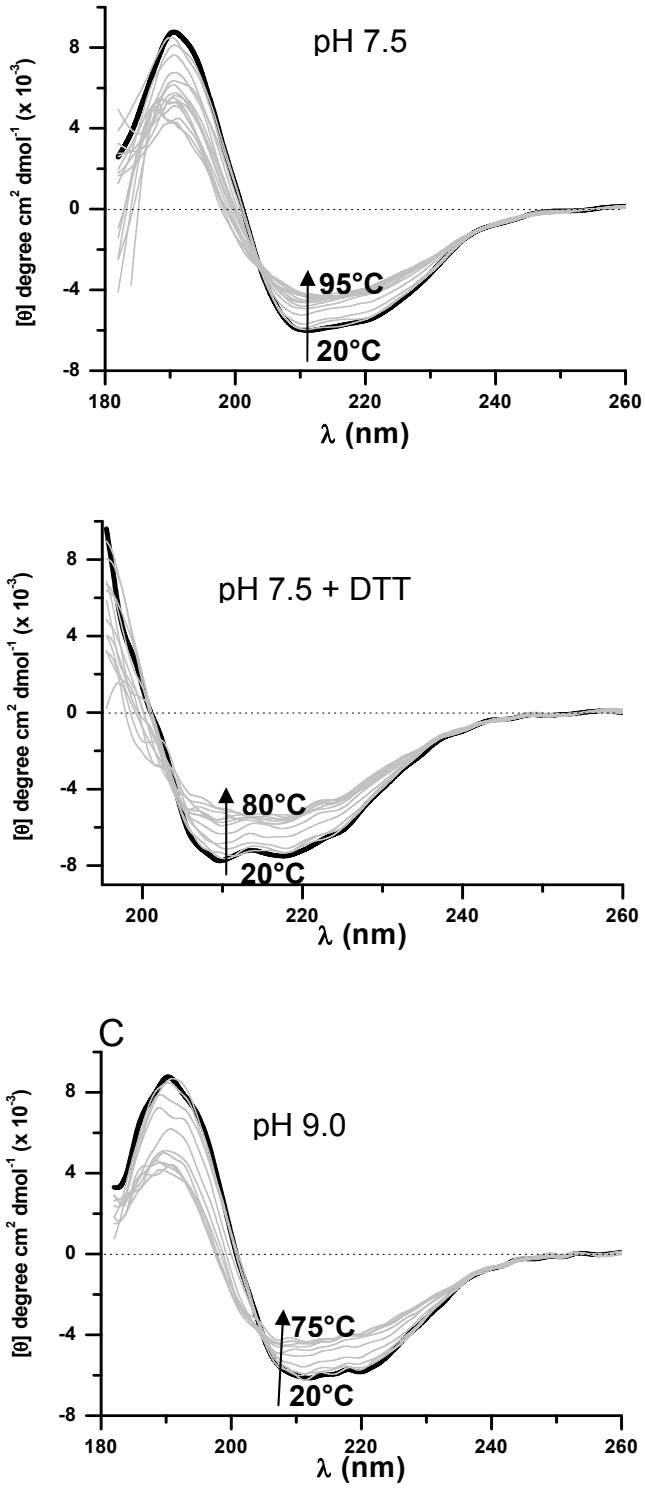


**FIGURE 1 Far-UV CD spectra of POPTb as a function of temperature.** a) Temperature ranging from 20 to 95 ºC at 2.5 mM Hepes pH 7.5, b) 20 to 80 ºC at 2.5 mM Hepes pH 7.5+DTT and from c) 20 to 75 ºC at 2.5 mM Tris-HCl pH 9.0.

*3.2 POPTb thermal unfolding*

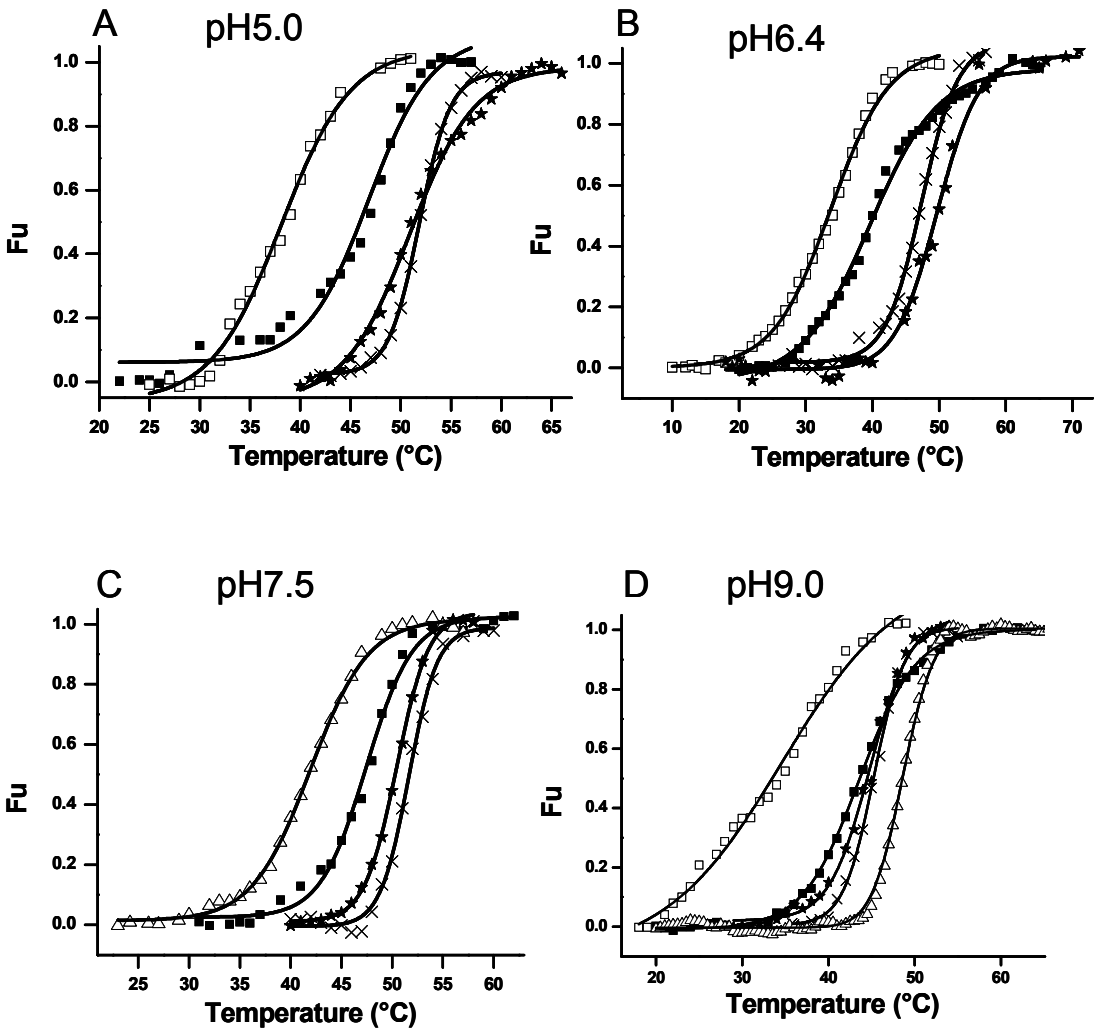


**FIGURE 2 Thermally-induced unfolding curves of POPTb obtained in the absence (■) or presence of 0.1 mM CTAB (□), 0.5 (☆) or 1 M (×) Sorbitol or 0.5 mM DTT (Δ).** A) pH 5.0; B) pH 7.5; C) pH 6.4; D) pH 9.0. These data were calculated considering the change in ellipticities at 218 nm. The estimated thermodynamic parameters derived from these analyses are presented in Table 1.

Based on the results from figure 1, we decided to further study the thermal denaturation of POPTb. Thermal unfolding experiments were carried out at pHs 5.0, 6.4, 7.5 and 9.0 in the presence or absence of the well-known stabilizing agents CTAB or

sorbitol or the reducing agent DTT. Figure 2 represents a summary of the experimental data obtained by recording the elipticities at 218 nm and fitting them according to Eq. 2.

**Table 1** – Thermodynamic parameters for the thermal unfolding of POPTb obtained from far-UV CD measurements at different pH values and in the presence or absence of additives.

| **Buffer** | **Tm (°C)** | **ΔHm (kcal.mol$^{-1}$)** | **ΔSm (kcal.mol$^{-1}$)** | **ΔG$^{25}$ (kcal.mol$^{-1}$)** |
|---|---|---|---|---|
| **Sodium Acetate pH 5.0** | 46.6 | 71.539 ± 4.201 | 224.1 ± 13.2 | 4.8 |
| + 0.1 mM CTAB | 38.6 | 59.638± 2.362 | 191.3± 7.5 | 2.6 |
| + 0.5 M Sorbitol | 51.1 | 79.321 ± 1.440 | 244.7 ± 4.4 | 6.4 |
| + 1M Sorbitol | 51.9 | 136.852 ± 660 | 421.2± 2.0 | 11.3 |
| **Sodium Cacodilate pH 6.4** | 40.0 | 53.907±828 | 172.2 ± 2.6 | 2.6 |
| + 0.1 mM CTAB | 35.0 | 42.902 ± 820 | 139.2 ± 2.7 | 1.4 |
| + 0.5 M Sorbitol | 49.9 | 81.339 ± 5.107 | 251.8 ± 15.8 | 6.3 |
| + 1 M Sorbitol | 47.5 | 89.234 ± 1206 | 278.4 ± 3.8 | 6.3 |
| **Hepes pH 7.5** | 47.2 | 92.100 ± 4.438 | 287.6 ± 13.9 | 6.4 |
| + 0.5 mM DTT | 41.8 | 69.942 ±1075 | 223.3 ± 3.4 | 3.7 |
| + 0.5 M Sorbitol | 50.5 | 116.522 ± 1.182 | 360.2 ± 3.7 | 9.2 |
| + 1 M Sorbitol | 51.7 | 137.323 ± 2.371 | 422.9 ± 7.3 | 11.3 |
| **TrisHCl pH 9.0** | 43.6 | 62.123 ± 589 | 200.1 ± 1.9 | 2.5 |
| + 0.5 M Sorbitol | 49.4 | 91.930± 2.150 | 285.2 ± 6.7 | 6.9 |
| + 1.0 M Sorbitol | 52.1 | 123.113 ± 1234 | 378.6 ± 3.8 | 10.3 |
| + 0.5 mM DTT | 48.6 | 128.189 ± 1.343 | 398.6 ± 4.2 | 9.4 |
| + 0.1 mM CTAB | 34.4 | 31.577± 1.265 | 103± 4.1 | 1.0 |

A plot of Fu versus temperature yields Tm, the transition midpoint temperature in which the amount of molecules in the native state equals that of molecules in the unfolded state for a monomeric protein in a two-state transition. The higher Tm values, 47.2 ºC and 46.6 ºC, (Fig. 2 and table 1) are observed at pH 7.5 and pH 5.0, respectively, when no stabilizing agents are added. Upon addition of 0.1 mM CTAB, however, the Tm values are lowered regardless of the pH. There is a 17 % reduction at pH 5.0 (Fig. 2A), 12.5 % at pH 6.4 (Fig. 2B), and 21 % at pH 9.0 (Fig. 2D). By contrast, in concentrations above 0.1 mM, the protein structure resists an increase in temperature and does not unfold, as evidenced by the preserved CD signal (data not shown). As for pH 7.5 in the presence of CTAB, the degree of denaturation was too small, making it hard to establish where denaturation starts or ends. Contrary to the effect of CTAB on POPTb thermal denaturation, sorbitol was responsible for an increase in the Tm, in spite of the pH value, by up to 9.9 °C (Fig. 2 and Table 1). DTT lowered the Tm by 5 °C at pH 7.5 and raised it by 5.4 °C at pH 9.0 (Fig 2 C and D and table 1).

*3.3 POPTb thermal stability*

The Tm values gave indications of the effects of additives and the pH on POPTb thermal unfolding. To better understand such process, thermodynamic parameters calculated from the van't Hoff equation were used. Figure 3 shows van't Hoff plots in which the slopes of the lines represent the standard enthalpy (ΔHm) changes of POPTb denaturation obtained in the presence of CTAB, Sorbitol and DTT in different pH values while the intercepts represent the standard entropy (ΔSm) changes. The values for ΔHm and ΔSm are given in Table 1. Regardless of the pH, while CTAB is able to decrease both ΔHm and ΔSm, sorbitol increases their values. DTT, on the other hand, decreases the

values of both parameters at pH 7.5 but increases them at pH 9.0. The effect of CTAB over these parameters at pH 7.5 could not be evaluated since the protein did not unfold in this condition.

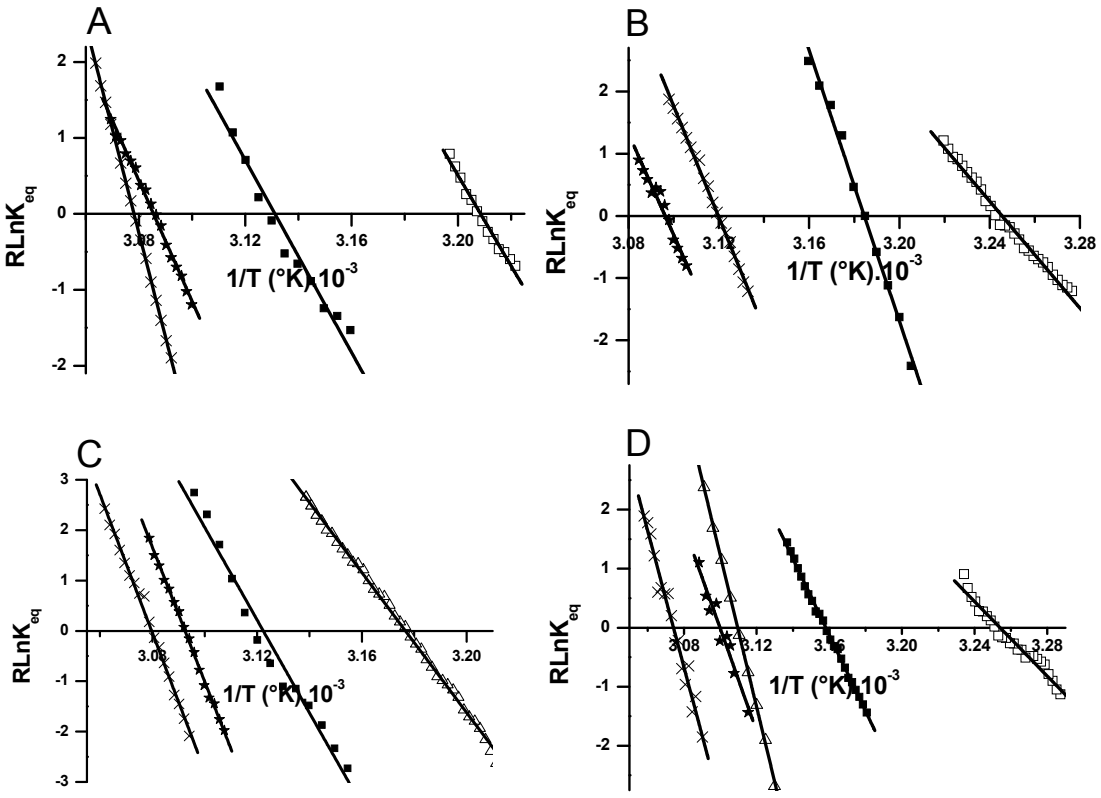


**FIGURE 3 Van`t Hoff plot of POPTb at different pHs.** The $K_{eq}$ constant values were obtained as described in Eq.4 (see Materials and Methods). The measurements were performed in the absence (■) or presence of 0.1 mM CTAB (□), 0.5 (★) or 1 M (×) Sorbitol or 0.5 mM DTT (Δ). A) pH 5.0; B) pH 7.5; C) pH 6.4; D) pH 9.0. The ΔH values were calculated for each sample from the slopes of van`t Hoff dependencies and are shown in Table 1.

These additives also alter the enzyme stability as shown in Figure 4 and Table 1. 0.1 mM CTAB destabilizes POPTb structure but sorbitol has an opposite effect as

indicated by the increase in $\Delta G^{25}$ values. At pH 7.5, CTAB prevents thermal denaturation from taking place. DTT decreases POPTb stability by 1.7 fold at pH 7.5 but increases it by almost 4 fold at pH 9.0. In the absence of additives, the enzyme is more stable at pH 7.5 as evidenced by $\Delta G^{25} = 6.4$ kcal.mol$^{-1}$ (Table 1).

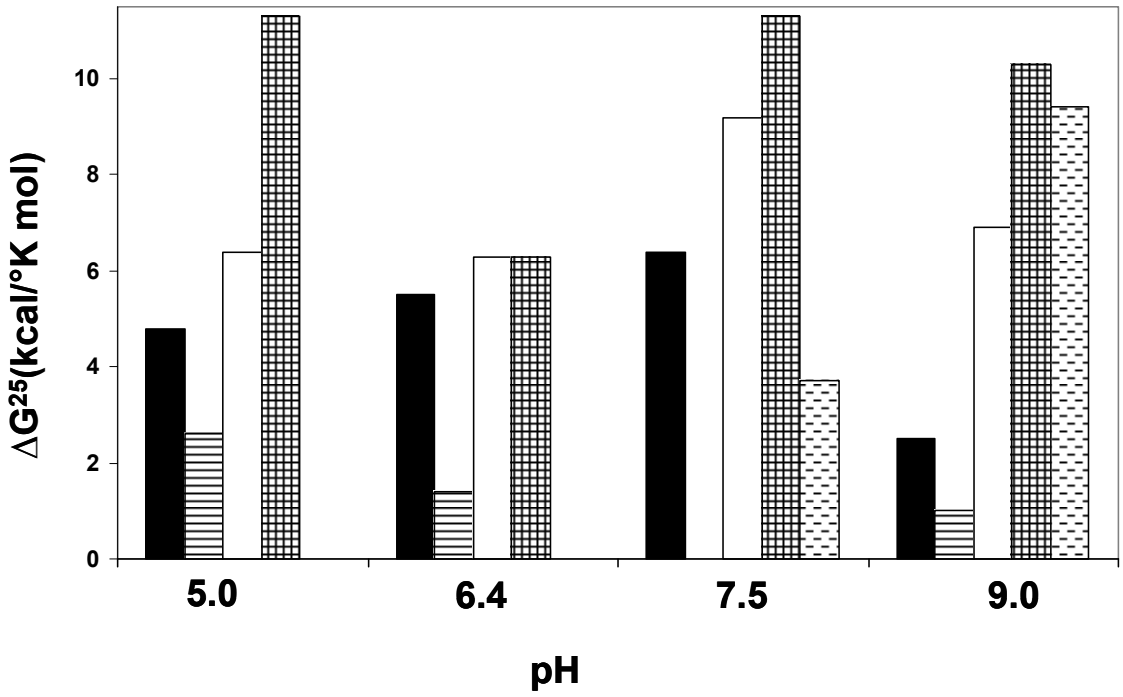


**FIGURE 4 Change of the free energy (ΔG) values as a function of pH and additives.** ΔG values were calculated according to Eq. 5 (Materials and Methods). No additive (■), 0.1 mM CTAB (≡), 0.5 M (□) or 1 M (#)Sorbitol or 0.5 mM DTT (---).

## 4. Discussion

Circular dichroism (CD) spectroscopy provides determinations of protein secondary structure and a way to rapidly assess conformational changes resulting from the addition of ligands. As the technique is relatively fast and can be performed on a small amount of sample, it has become the method of choice for studying protein folding, conformational stability and structural changes induced by temperature and ligand binding [12-14]. The thermally induced denaturation of POPTb in the absence or presence of additives was studied by CD spectroscopy. POPTb stability can be significantly enhanced by the addition of sorbitol. Such effect is in agreement with earlier reports which have demonstrated sorbitol was effective in protecting proteins against thermal inactivation [15-17]. This result is important for our long-term goal of drug design since we can use sorbitol in the crystallization experiments that will be carried out.

CTAB is known to stabilize proteins through an effective confinement by micellization [18]. 0.1 mM CTAB reduced the thermal stability of POPTb. These results are in agreement with chemical denaturation experiments of POPTb which demonstrated a stabilizing and a destabilizing effect for Sorbitol and CTAB, respectively [19]. In concentrations above 0.1 mM, the cationic surfactant did not allow for the thermal denaturation of the protein to take place, regardless of the pH. This effect is expected at and above the CMC (critical micelle concentration), namely 1 mM for CTAB [20]. However, our results suggest that the increase in temperature lowers the cmc, possibly due to relatively weak electrostatic interactions at high temperatures.

DTT is frequently used to reduce disulfide bonds of proteins. The reduction is carried out under denaturing conditions if the bonds are buried in the protein structure. Furthermore, it is used to prevent intramolecular and intermolecular disulfide bonds

between cysteine residues of proteins. Since the reducing power of DTT is limited to pH values above approximately 7, we conducted our experiments in neutral and alkaline pH. In the former, DTT reduced enzyme stability while in the latter it was able to increase the stability by a factor of almost 4. Since POPTb is predicted to have 8 cysteine residues, these results could indicate the participation of disulfide cross-links in the structure of the enzyme. Our previous results suggest that substrate access to the catalytic pocket of POP takes place in the vicinity of both domains, requiring some flexibility of the structure [5].

Chemical denaturation studies of POPTb we carried out previously [19] demonstrated low values for Gibbs free energy. Guanidine hydrochloride (GuHCl) and urea unfolding assays yielded $\Delta G^{25}_{H2O}$ of 3.60 ± 0.27 and 3.70 ± 0.44 kcal.mol$^{-1}$, respectively, which led us to conclude POPTb was not a very stable enzyme. However, heat-induced denaturation experiments yielded a much higher value ($\Delta G^{25}$ = 6.4 kcal.mol$^{-1}$). The divergence between these results and the fact that the protein did not unfold completely under the conditions tested suggest that all the calculated parameters represent the unfolding of the protein up to an intermediate state. It suggests, as well, that POPTb is not as unstable an enzyme as earlier thought. It is possible that the protein domains are different and present such a degree of organization that allows them to unfold independently. Indeed, POP family members are shown to be structurally organized as multidomain proteins displaying a peptidase domain and a seven-bladed β-propeller domain [3]. Therefore, the calculated $\Delta G^{25}$ values are a partial representation of the break of non-covalent bonds which maintain the domains of the enzyme.

To better understand this process and the changes that it entails, differential scanning calorimetry studies will be carried out along with thermal denaturation experiments in high ionic strength conditions.

In this work, we present the characterization of the pH dependence on *T. brucei* POP thermal stability. Sorbitol increases enzyme stability and 0.1 mM CTAB decreases it. DTT, however, has the former effect in alkaline pH and the latter effect in neutral pH. POPTb was not fully unfolded under the conditions tested indicating its structural stability might be higher than previously expected.

**Acknowledgements**

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico CNPq, Fundação de Amparo à Pesquisa do Distrito Federal FAPDF, Financiadora de Estudos e Projetos FINEP, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior CAPES and Associação Brasileira de Tecnologia de Luz Síncrotron ABTLuS. MML and IMDB are fellows of CNPq and CAPES, respectively

## 5. References

[1] O.V. Nikolskaya, A.P.A. Lima, Y.V. Kim, J.D. Lonsdale-Eccles, T. Fukuma, J. Sharfestein, D. Grab, Blood-brain barrier traversal by African trypanosomes requires calcium signaling induced by parasite cysteine protease, J. Clin. Investig. 116 (2006) 2739-2747.

[2] M. Reincke, W. Arlt, C. Heppner, F. Petzke, G.P. Chrousos, B. Allolio, Neuroendocrine dysfunction in African trypanosomiasis. The role of cytokines, Ann. N. Y. Acad. Sci. 840 (1998) 809-821.

[3] V. Fülop, Z. Böcskei, L. Polgár, Prolyl oligopeptidase: an anusual β–propeller domain regulates proteolysis, Cell 94 (1998) 161-170.

[4] P. Grellier, S. Vendeville, R. Joyeau, I.M.D. Bastos, H. Drobecq, F. Frappier, A.R.L. Teixeira, J. Schrével, E. Davioud-Charvet, S. Sergheraert, J.M. Santana, *Trypanosoma cruzi* prolyl oligopeptidase Tc80 is involved in nonphagocytic mammalian cell invasion by trypomastigotes, J. Biol. Chem. 276 (2001) 47078-47086.

[5] I.M.D. Bastos, P. Grellier, N.F. Martins, G. Cadavid-Restrepo, M.R. de Souza-Ault, K. Augustyns, A.R.L. Teixeira, J. Schrével, B. Maigret, J.F. da Silveira, J.M. Santana, Molecular, functional and structural properties of the prolyl oligopeptidase of *Trypanosoma cruzi* (POP Tc80) that is required for parasite entry into mammalian cells, Biochem. J. 388 (2005) 29-38.

[6] J.A. García-Horsman, P.T. Männisto, J.I. Venäläinen, On the role of prolyl oligopeptidase in health and disease, Neuropeptides 41 (2007) 1-24.

[7] S.M. Kelly, T.J. Jess, N.C. Price, How to study proteins by circular dichroism, Biochim.Biophys. Acta 1751 (2005) 119-139.

[8] A.J. Dowd, J.P. Dalton, A.C. Loukas, P. Prociv, P.J. Brindley, Secretion of cysteine proteinase activity by the zoonotic hookworm *Ancylostoma caninum*, Am. J. Trop. Med. Hyg. 3 (1994) 341-347.

[9] J.L.Kinsella, R.J. Lichtenfels, M.F. Ryan, A preliminary analysis of proteolytic activity of excretory–secretory products from Cyathostominea, Vet. Parasit. 107 (2002) 73–83.

[10] A.A. De Sa Pinheiro, J.N. Amazonas, F.S. Barros, L.F. Menezes, E.J.O. Batista, E.F. Silva, W. Souza, J.R. Meyer-Fernandes, *Entamoeba histolytica*: An ecto-phosphatase activity regulated by oxidation–reduction reactions, Exp. Parasit. 115 (2007) 352–358.

[11] R.E. Morty, J.D. Lonsdale-Eccles, J. Morehead, E.V. Caler, R. Mentele, E.A. Auerswald, T.H. Coetzer, N.W. Andrews, B.A. Burleigh, Oligopeptidase B from *Trypanosoma brucei*, a new member of an emerging subgroup of serine oligopeptidases, J. Biol. Chem. 274 (1999) 26149–26156.

[12] J.T. Pelton, L.R. McLean, Spectroscopic methods for analysis of protein secondary structure, Ann. Biochem. 277 (2000) 167-176.

[13] J.G. Lees, B.A. Wallace, Synchrotron radiation circular dichroism and conventional circular dichroism spectroscopy: A comparison, Spectroscopy 16 (2002) 121–125.

[14] D. Andersson, U. Carlsson, P.O. Freskgard, Contribution of tryptophan residues to the CD spectrum of the extracellular domain of human tissue factor, Application in folding studies and prediction of secondary structure, Eur. J. Biochem. 268 (2001)1118-1128.

[15] T. De Diego, P. Lozano, S. Gmouh, M. Vaultier, J. L. Iborra, Fluorescence and CD spectroscopic analysis of the α-chymotrypsin stabilization by the ionic liquid, 1-ethyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]amide, Biotechnol. Bioeng. 88 (2004) 916-924.

[16] G. Xie, S.N. Timasheff, Mechanism of the stabilization of ribonuclease A by sorbitol: Preferential hydration is greater for the denatured than for the native protein, Protein Sci. 6 (1997) 211-221.

[17] R. Di Domenico, R. Lavecchia, Thermal stability of human haemoglobin in the presence of sarcosine and sorbitol, Biotechnol. Lett. 22 (2000) 335-339.

[18] D. Lu, A. Liu, J. Wu, Structural transitions of confined model proteins: molecular dynamics simulation and experimental validation, Biophys. J. 90 (2006) 3224-3238.

[19] M.M. Lima, I.M.D. Bastos, D. Neves, F.J. Medrano, J.A.R.G. Barbosa, P. Grellier, S.M. Freitas, J.M. Santana, Structural stability of *Trypanosoma brucei* prolyl oligopeptidase.

[20] J. Mansfeld, R. Ulbrich-Hofmann, The stability of engineered thermostable neutral proteases from Bacillus stearothermophilus in organic solvents and detergents, Biotechnol. Bioeng. 97 (2007) 672-679.

# CONCLUSÕES

**“Science is an imaginative adventure of the mind seeking truth in a world of mystery.”**
**Sir Cyril Herman Hinshelwood**

# CONCLUSÕES

Este trabalho teve como objetivo a caracterização biofísica da enzima prolil oligopeptidase do protozoário *Trypanosoma brucei*, agente etiológico da doença do sono. Os dados obtidos permitem as seguintes conclusões:

- Cloreto de guanidina é desnaturante mais forte que uréia;
- Sorbitol tem ação estabilizante sobre a estrutura da enzima;
- CTAB age desestabilizando a estrutura da enzima;
- Altas temperaturas reduzem o valor da cmc do CTAB
- A molécula da protease está menos sujeita a alterações conformacionais em pH 7.5;
- O ambiente dos triptofanos é heterogênio e ao menos um resíduo deve estar localizado nas proximidades do sítio ativo;
- Ação de sorbitol é independente do pH;
- Ação do DTT é pH-dependente;
- Intermediários parecem ser formados durante o processo de desdobramento da enzima;
- A estabilidade da enzima parece ser maior do que incialmente imaginado.

# PERSPECTIVAS

**“Science knows no country, because knowledge belongs to humanity, and it is the torch which illuminates the world.”**
**Louis Pasteur**

# PERSPECTIVAS

Todos os dados disponíveis acerca da doença do sono – severidade da doença, toxicidade e ineficácia das drogas disponíveis, número de casos, dentre outros – apontam para a necessidade premente de novos fármacos a fim de tratar a infecção pelo *T. brucei*. Partindo-se do princípio que um bom alvo de droga seja uma proteína necessária ao parasito e cujo homólogo no hospedeiro, se houver, seja suficientemente diferente, acreditamos que a POPTb reúna essas características. Por essa razão, ela foi enquadrada na linha de pesquisa do nosso laboratório que busca identificar e caracterizar potenciais alvos para o desenho racional de drogas.

Uma etapa essencial para alcançar esse objetivo é a resolução da estrutura tridimensional da enzima. É necessário conhecer a localização espacial dos resíduos que compõem o sítio ativo. Experimentos de cristalização estão em andamento e alguns cristais já foram obtidos. Entretanto, seu número e qualidade não foram suficientes para a coleta de dados. Novos experimentos de *screening* e refinamento das melhores condições são o próximo passo. A estrutura tridimensional da enzima nos permitirá conduzir varredura *in silico* com o objetivo de encontrar moléculas com possíveis propriedades inibitórias que poderiam servir de base para outros estudos visando a obtenção de novos biofármacos. Enquanto isso, pretendemos empregar modelagem molecular por homologia de seqüência para obter um modelo de estrutura que possa ser empregado na busca por possíveis inibidores. Em nosso laboratório, esse procedimento já está em andamento utilizando a estrutura da POPTc80 e com resultados promissores.

Paralelamente, vamos conduzir experimentos de calorimetria exploratória diferencial (DSC) e desnaturação térmica na presença de alta força iônica na tentativa de determinar a razão da discrepância observada na estabilidade da enzima durante desnaturação térmica e química.

# REFERÊNCIAS

# BIBLIOGRÁFICAS

**“To do successful research, you don’t need to know everything, you just need to know of one thing that isn’t known.”**
**Arthur Schawlow**

## REFERÊNCIAS BIBLIOGRÁFICAS


1. Barrett, M.P., Boykin, D.W., Brun, R. & Tidwell, R.R. (2007). Human African trypanosomiasis: pharmacological re-engagement with a neglected disease. Br. J. Pharmacol. **152**, 1155 – 1171.
2. Bastos, I.M.D. Prolil oligopeptidases de Trypanosoma cruzi e Trypanosoma brucei compartilham propriedades genéticas, estruturais e funcionais. (2003). Tese de Doutorado, Universidade de Brasília.
3. Bastos, I.M.D., Grellier, P., Martins, N.F., Cadavid-Restrepo, G., De Souza-Ault, M.R., Augustyns, K., Teixeira, A.R.L., Schrével, J., Maigret, B., Da Silveira, J.F. & Santana, J.M. (2005). Molecular, functional and structural properties of the prolyl oligopeptidase of *Trypanosoma cruzi* (POP Tc80), which is required for parasite entry into mammalian cells. Biochem. J. **388**, 29-38.
4. Brandt, I., Vriendt, K., Devreese, B., Beeumen, J., Dongen, W., Augustyns, K., Meester, I., Scharpé, S. & Lambeir, A.M. (2005). Search for substrates for prolyl oligopeptidase in porcine brain. Peptides **12**, 2536-2546.
5. Brun, R. & Balmer, O. (2006). New developments in human African trypanosomiasis. Curr. Opin. Infect. Dis. **19**, 415-420.
6. Eftink, M.R. Fluorescence Quenching: Theory and Applications, in: Topics in Fluorescence Spectroscopy, volume 2: Principles, J. R. Lakowicz, editor. Plenum Press, New York, 1991, pp. 53-121.
7. Fülop, V., Böcskei, A. & Polár, L. (1998). Prolyl oligopeptidase: an anusual β–propeller domain regulates proteolysis. Cell **94**, 161-170.
8. Fülop, V., Szeltner, Z. & Polgár, L. (2000). Catalysis of serine oligopeptidases is controlled by a gating filter mechanism. EMBO Rep. **1**, 277-281.
9. Grellier, P., Vendeville, S., Joyeau, R., Bastos, I.M.D., Drobecq, H., Frappier, F., Teixeira, A.R.L., Schrével, J., Davioud-Charvet, E., Sergheraert, S., Santana, J.M. (2001). *Trypanosoma cruzi* prolyl oligopeptidase Tc80 is involved in nonphagocytic mammalian cell invasion by trypomastigotes, J. Biol. Chem. **276**, 47078-47086.
10. Hausch, F., Shan, L., Santiago, N.A., Gray, G.M. & Khosla, C. (2002). Intestinal digestive resistance of immunodominant gliadin peptides. AJP-Gastrointest liver Physiol. **283**, G992-1003.

11. Hellemond, J.J. & Tielens, A.G.M. (2006). Adaptations in the lipid metabolism of the protozoan parasite *Trypanosoma brucei*. FEBS Letters **580**, 5552-5558.
12. Hope, J., Shearman, M.S., Baxter, H.C., Chong, A., Kelly, S.M.& Price, N.C. (1996). Cytotoxicity of prion protein peptide (PrP106-126) differs in mechanism from the ytotoxic activity of the Alzheimer's disease amyloid peptide Aß 25-35. Neurodegeneration **5**, 1-11.
13. Hughes, K., Wand, M., Foulston, L., Young, R., Harley, K., Terry, S., Ersfeld, K. & Rudenko, G. (2007). A novel ISWI is involved in VSG expression site downregulation inAfrican trypanosomoses. EMBO Journal **26**, 2400-2410.
14. Irazusta, J., Larrinaga, G., Gonzalez-Maeso, J. Gil, J., Meana, J.J. & Casis, L. (2002). Distribution of prolyl endopeptidase activities in rat and human brain. Neuroche. Int. **40**, 337-345.
15. Kelly, S.H., Jess, T.J. & Price, N.C. (2005). How to study proteins by circular dichroism. Biochim. Biophys. Acta **1751**, 119-139.
16. Kelly, S.M.& Price, N.C. (1997). The application of circular dichroism to studies of protein folding and unfolding. Biochim. Biophys. Acta **1338**, 161-185.
17. Kennedy, P.G.E. (2006). Diagnostic and neuropathogenesis issues in human African trypanosomiasis. Int. J. Parasit. **36**, 505-512.
18. Kowal, N.W., Beal, M.F., Busciglio, J., Duffy, L.K. & Yanker, B.A. (1991). An in vivo model for the neurodegenerative effects of beta amyloid and protection by substance P. Proc. Nat. Acad. Sci. **88**, 7247-7251.
19. Lakowicz, J.R. Protein Fluorescence, in: Principles of Fluorescence Spectroscopy. Plenum Press, New York, 1983, pp. 341-379.
20. Lee, S.H., Stephens, J.L., Paul, K.S. & Englund, P.T. (2006). Fatty acid synthesis by elongases in trypanosomes. Cell **126**, 691-699.
21. Morain, P., Lestage, P., De Nanteuil, G., Jochemsen, R., Robin, J.L., Guez, D. & Boyer, P.A. (2002). S 17092: a prolyl endopeptidase inhibitor as a potential therapeutic drug for memory impairment. Preclinical and clinical studies. CNS. Drug. Rev. **8**, 31-52.
22. Nandi, P.K., Leclerc, E. & Marc, D. (2002). Unusual property of prion protein unfolding in neutral salt solution. Biochemistry **41**, 11017-11024.
23. Polgár, L. (2002). The prolyl oligopeptidase family. Cell. Mol. Life. Sci. **50**, 349-362.

24. Price, N.C. (2000). Conformational issues in the characterization of proteins. Biotechnol. Appl. Biochem. **31**, 29040.
25. Rawlings, N.S., Polgár, L. & Barrett, A.J. (1991). A new family of serine-type peptidases related to prolyl oligopeptidase. Biochem. J. **279**, 907-908.
26. Santana, J.M., Grellier, P., Schrével, J., Teixeira, A.R.L. (1997). A *Trypanosoma cruzi*-secreted 80 kDa proteinase with specificity for human collagen types I and IV. Biochem. J. **324**, 129-137.
27. Shan, L., Mathews, I.I. & Khosla, C. (2005). Structural and mechanistic analysis of two prolyl endopeptidases: Role of interdomain dynamics in catalysis and specificity. Proc. Nat. Acad. Sci. **102**, 3599-3604.
28. Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., gray, G.M., Solid, L.M. & Khosla, C. (2002). Structural basis for gluten intolerance in celiac sprue. Science **297**, 2275-2279.
29. Shiflett, A.M., Faulkner, S.D., Cotlin, L.F., Widener, J.W., Stephens, N. & Hajduk, S.L. (2007). African trypanosomes: Intracellular trafficking of host defense molecules. J. Eukaryot. Microbiol. **54**, 18-21.
30. Sultan, N.A.M., Rao, R.N., Nadimpalli, S.K., Swamy, M.J. (2006). Tryptophan environment, secondary structure and thermal unfolding of the galactose-specific seed lectin from *Dolichos lablab*: Fluorescence and circular dichroism spectroscopic studies. Biochim. Biophys. Acta **1760**, 1001-1008.
31. Szeltner, A., Rea, D., Juhász, T., Renner, V., Fülop, V. & Polgár, L. (2004). Concerted structural changes in the peptidase and the propeller domains of prolyl oligopeptidase are required for substrate binding. J. Mol. Biol. **340**, 627-637.
32. Venäläinen, J.I., Garcia-Horsman, J.A., Forsber, M.M., Jalkanen, A., Wallén, E.A.A., Jarho, E.M., Christiaans, J.AM., Gynther, J. & Männistö, P.T. (2006). Binding kinectics and duration of in vivo action of novel prolyl oligopeptidase inhibitors. Biochem. Pharmacol. **71**, 683-692.
33. World Health Organization: African trypanosomiais (sleeping sickenss). *World Health Organ Fact Shee*t 2006, **259**: [http://www.who.int/mediacentre/factsheets/fs259/en/].
34. Yoshimoto, T., Kado, K., Matsubara, F., Koriyama, N., Kaneto, H., Tsura, D. (1987). Specific inhibitors for prolyl endopeptidase and their anti-amnesic effect. J. Pharmacobiodyn. **10**, 730-735.

# ANEXO

**“The scientist is not a person**
**who gives the right answers,**
**he's one who asks the right questions.”**
**Claude Lévi-Strauss,**

# ANEXO

## 1. The drug target methylthioadenosine phosphorylase of Trypanosoma cruzi exhibits remarkable resistance to thermal and chemical denaturation.

Trabalho realizado em colaboração durante o doutorado

**THE DRUG TARGET METHYLTHIOADENOSINE PHOSPHORYLASE of *Trypanosoma cruzi* EXHIBITS REMARKABLE RESISTANCE TO THERMAL AND CHEMICAL DENATURATION.**

David Neves[1], Meire Maria de Lima[1], Izabela Dourado Bastos[1], Gloria Restrepo-Cadavid[1], Sônia Freitas[2], Francisco Javier Medrano[3], João Alexandre R. G. Barbosa[4], Philippe Grellier[5] and Jaime Martins Santana[1].

[1] Laboratório de Interação Parasito-Hospedeiro, Faculdade de Medicina, Universidade de Brasília, Brazil

[2]Laboratório de Biofísica, Instituto de Ciências Biológicas, Universidade de Brasília, Brazil

[3]Universidade Estadual de Campinas, Campinas, Brazil

[4]Centro de Biologia Molecular Estrutural, Laboratório Nacional de Luz Síncrotron, Campinas, Brazil

[5] USM 504 Muséum National d'Histoire Naturelle, Paris, France

***Corresponding authors.** Laboratório de Interação Parasito-Hospedeiro, Faculdade de Medicina, Universidade de Brasília, 70.910-900 Brasília DF, Brazil; e-mail: jsantana@unb.br; phone (55) 61 33072256.

**SUMMARY**

Methylthioadenosine phosphorylase (MTAP) belongs to the family 2 of purine nucleoside phosphorylase/MTAP, whose members assembles into different oligomeric constitutions. These widely distributed enzymes catalyze the phosphorolysis of MTA, a byproduct of polyamine biosynthesis and are the starting point of purine and methionine salvage pathways. Herein, we report the physicochemical characterization of the MTAP of the trypanosomatid *Trypanosoma cruzi* (TcMTAP) that is considered a potential target for chemotherapy of Chagas disease, an incurable sickness responsible for thousands of deaths in Latin America. Thermal denaturation followed by circular dichroism performed at broad range of pH showed reduced dependence between enzyme stability and environment ionization condition. TcMTAP exhibited striking resistance to thermal denaturation, unfolding only above 79 °C. Its secondary structure content is significantly composed of α-helices, as predicted from circular dichroism data. However, even after thermal denaturation, the content of α-helices showed little variation, markedly at pH 7.4 and 9.0. Chemical denaturation of TcMTAP, induced by guanidine-hydrochloride or urea, was monitored by far-UV circular dichroism and fluorescence spectroscopy. The enzyme kept folded in concentration as high as 3.6 M of guanidine-hydrochloride and 8 M of urea. The thermodynamic parameters calculated from thermal and chemical denaturation assays were in good agreement, $\Delta G(H_2O)$ = 35.5 kcal/mol and $\Delta G^{25}$ = 36 kcal/mol. Thermal denaturation carried out in the presence of hexadecyltrimethylammonium (CTAB) increased enzyme stability at pH 6.0. Knowledge of biophysical properties of TcMTAP may aid in understanding its structural behavior in different environment and in interpreting its peculiar biochemical characteristics, thus helping in development of specific inhibitors.

## INTRODUCTION

Methylthioadenosine phosphorylase (MTAP; E.C. 2.4.2.28) belongs to the purine nucleoside family 2 (PNP, E.C. 2.4.2.1) and is ubiquitously distributed in nature. It phosphorolytically cleaves the glycosidic bond of the sulfur-containing nucleoside methylthioadenosine (MTA), which is a metabolic product of S-adenosylmethionine (AdoMet) in polyamine biosynthesis. MTA is also the starting point of two major salvage pathways, the purine and methionine ones [1]. MTAP readily metabolizes MTA to adenine and methylthio-ribose-1-phosphate (MTR1P). Adenine will ultimately replenish the AMP and ATP pool whereas MTR1P will be converted to methionine. The MTAP and the intermediates of the pathways associated with MTA recycling have been considered chemotherapy targets against human cancers related with alterations at the chromosomal locus 9p21 and against protozoan diseases such as trypanosomiasis, leishmaniasis and malaria. This is because those cancer cells and protozoa parasites lack the machinery to synthesize the purine ring de novo and, thus, depend on the salvage pathway [2,3]. Differently from human MTAP, that show high specificity for MTA, MTAPs of parasitic protozoa also displays activity on other nucleosides and its analogues [4]. For instance, it has been shown that *Trypanosoma brucei* and *Trypanosoma rhodesiense* are susceptible to the MTA analog, 5'-deoxy-5'-(hydroxyethylthio)adenosine (HETA), which was able to cure trypanosoma-infected mice without damaging mammalian cells [5,6].

Thermodynamic characterization may contribute to explain enzyme's biochemical properties, for example a broad pH range activity, thermostability, and substrate preference [7]. In addition, the biophysical data can also provide insights into the molecular mechanisms related with protein stability, overall structure and biotechnological applicability [8], e. g. protein-ligand affinities, presence of intermediate in the folding/unfolding process and rational drug design [9,10].

The kinetoplastid protozoan *Trypanosoma cruzi* is the etiological agent of Chagas disease, which is a chronic infection highly prevalent in Latin America. The drugs currently used to treat human *T. cruzi* infection neither prevent the chronic phase of the disease nor eliminate the parasite. Although new potential drugs are being evaluated there is an urge to characterize *T. cruzi* virulence factors that could be used as targets for the conception of new molecules to treat this parasitic infection [11]. Purine salvage pathway

of *T. cruzi* is considered a feasible drug target since the parasite, likewise other kinetoplastids, is unable to synthesize the purine ring de novo [4,12,13]. We have biochemically characterized the recombinant purine-salvage-pathway participating enzyme MTAP of *T. cruzi* (rTcMTAP) [14]. The enzyme is active over a broad pH range, displays considerable thermostability and is capable to renature after chemical unfolding. rTcMTAP assembles into oligomers in solution, which apparently is composed by a mixture of association species [14]. Similar results were found by, Wielgus-Kutrowska [15], on *Cellulomonas* sp. PNP.

In this study we calculated the thermodynamic parameters and characterized the structural stability of the recombinant MTAP from *T. cruzi*. rTcMTAP exhibited striking resistance to thermal and chemical denaturation, followed by circular dichroism (CD) and fluorescence spectroscopy. The enzyme unfolds as a two-state model and increases its thermal stability at acid to neutral pH. In addition, the secondary structure content suffers little variation during the thermal unfolding process. We also tested the presence of stabilizers hexadecyltrimethylammonium bromide (CTAB) and sorbitol. which altered the enzyme stability and its denaturation pattern. Until present date, only the *Cellulomonas* sp. PNP [15] had its biophysical parameters fully determined, but no member of MTAP sub-family.

## RESULTS

### Thermal stability

Far-UV circular dichroism spectra of rTcMTAP at pH ranging from 4.0 to 9.0, recorded during thermal denaturation, show maximum peak at 192 nm and dichroic bands at 208 and 222 nm (Fig. 1). In addition, the protein spectra pattern, even after the unfolding transition, kept similar to the native, markedly at pH 7.4 and 9.0. To confirm this hypothesis, the secondary structure content was predicted [16] at 20 and 95 °C for all pH (Table 1) using the CDPro software package. The α-helix content showed modest decrease after denaturation, more strikingly at pH 7.4 and 9.0. These data indicate the secondary structure content of rTcMTAP is formed predominantly of α-helix and that thermal denaturation has diminute effect over its content.

The thermal unfolding, which was monitored at 208 nm, appeared as a single transition at pH 4.0, 5.2, 6.0, and 7.4 (Fig. 2) and its data were fitted as a nonlinear extrapolation, according to the van't Hoff approximation [17]. We observed a sigmoid

dependence of the ellipticity with the temperature, with practically no alteration until 60 °C, followed by a large change in the ellipticity, which characterized the unfolding process (Fig. 2). By these results, we assumed that rTcMTAP unfolding process follows a two-state model since the shape of the transition curve is characteristic of a highly cooperative process. The sharp transition curves resulted in melting temperatures ($T_m$) equal or slightly greater than 79.8 °C (Fig. 2 and Table 2), which were extrapolated from the fitted transition curves. The highest stability at 25 °C ($\Delta G^{25}$) in the absence of additives was at pH 7.4, however its value was very similar to the others pH conditions (Table 2). We also performed thermal denaturation at pH 7.4 in the presence of phosphate, a MTAP substrate, to evaluate its effect on enzyme stability. The $\Delta G^{25}$ value almost doubled, from 36 to 61.2 kcal/ mol, when it was added. At pH 4.0, the enzyme showed lower $\Delta G^{25}$ when compared to 5.2, 6.0 and 7.4. Although significant differences were observed in $\Delta G^{25}$, $T_m$ values were similar, ranging from 79.8 °C at pH 4.0 to 81.2 °C at pH 7.4.

We also investigated the effect of higher pH over rTcMTAP stability. Although the CD spectra indicated that at pH 9.0 the environment had modest effect over enzyme secondary structure (Fig. 1E, Table 1), the thermal denaturation at pH 8.0, 8.6 and 9.0 exhibited an altered transition slope (Fig. 3). Since the shape of the denaturation slopes were not characteristic of a two-state model transition the thermodynamic parameters could not be calculated for these conditions.

To determine the effects of known protein stabilizers on rTcMTAP, we incubated the enzyme with CTAB or sorbitol at pH 6.0 and 7.4. The thermodynamic parameters were determined (Table 2) and the denaturation curves in the presence (0.1, 0.5 and 1.0 mM) of detergent are shown in Fig. 2 (C and D, respectively). At pH 6.0, CTAB strikingly increased rTcMTAP stability. For instance, in the presence of 0.5 mM CTAB the value of $\Delta G^{25}$ triplicate and $T_m$ changed to 97.6 °C. At pH 7.4, CTAB increased the enzyme melting temperature (Table 2), however it became the unfolding reaction less cooperative, as judged by the denaturation curves (Fig. 2D). Unfolding slopes were not observed when the assay was performed in the presence of 1.0 mM of CTAB or sorbitol (0.5 and 1.0 M) at both pHs (data not shown).

Since the presence of CTAB and sorbitol altered the enzyme stability, we determined if they were also changing the protein structure by recording CD and fluorescence spectra. CTAB red-shifted the fluorescence maximum emission wavelength

from 333 to 339 nm and also increased the fluorescence intensity as shown in Fig 4A. However, CTAB at different pH did not induce significant changes in the CD spectra profile (data not shown). In opposite, sorbitol affected the CD spectra, increasing the signal intensity at pH 6.0 (data not shown) and 7.4 (Fig. 4B). However, it did not alter the emission bands position in the fluorescence spectra, although the signal intensity was quenched (Fig. 4C). The linear extrapolation slopes, employed to calculate $\Delta H_u$ and $\Delta S_u$, evidenced the CTAB effect over this two parameters (Fig. 5). For instance, at pH 7.4 (Fig. 5, open symbols), CTAB drastically altered the slopes angle, but remained close to each other, reflecting mainly in the alteration of $\Delta H_u$ value.(Table 1). At pH 6.0, CTAB increased $\Delta H_u$ and $\Delta S_u$ values (Table 2), where the $\Delta S_u$ change is evidenced by the distance among the slopes (Fig. 5, filled symbols).

**Chemical Equilibrium**

Perturbations in the micro-environment of the two tryptophans residues, at positions 167 and 230 of TcMTAP sequence, were evaluated by increasing concentrations of Gnd-HCl and urea followed by fluorescence spectroscopy (Fig. 6). The native rTcMTAP fluorescence spectrum showed a maximum wavelength at 335 nm (Fig. 6 A and B, top spectrum). During Gnd-HCl denaturation, emission spectra positions were preserved with decrease in intensity up to 3.6 M. Thereafter, higher concentrations of Gnd-HCl induced both red-shifting and decreasing in intensity of emission bands. In contrast to the folded state, at 7 M of Gnd-HCl, rTcMTAP emission spectrum adopted maximum wavelength at 352 nm and its intensity dropped to about 45% of that measured for the folded enzyme, characterizing exposure of tryptophans to the solvent. These data suggest that the tryptophans residues are buried in a hydrophobic environment in the folded structure [18] that maintains its native conformation in the presence of Gnd-HCl concentrations as high as 3.6 M. We also recorded rTcMTAP ellipticities at the same experimental conditions. Increasing concentrations of Gnd-HCl induced a sharp unfolding transition curve (Fig. 6C), corroborating the thermal denaturation results (Fig. 2). The stabilization free energy value in absence of Gnd-HCl ($\Delta G_u^{H2O}$) calculated from CD data was 35.5 kcal /mol. In addition to the remarkable resistance to Gnd-HCl, rTcMTAP resisted to urea concentrations as high as 8 M without showing modification in the maximum wavelength band position that could characterize an unfolding process. However, the band intensity decreased in higher concentrations of the urea (Fig. 6B).

Likewise the fluorescence assay, the CD ellipticities in the presence of urea did not exhibit a transition pattern (data not shown).

## DISCUSSION

Equilibrium denaturation studies on the folding/unfolding of proteins have largely been done on small monomeric globular proteins. Nevertheless, analysis of the forces and energies involved in the folding and packing of oligomeric proteins could provide additional information on subunit interactions and in the folding process [7-9,19,20]. The main difference between mono and oligomeric folding is the existence of interactions between the protein subunits. Consequently, the intra and intermolecular interactions of the folding pathway must be taken into account since it will influence the overall stability and integrity of oligomeric proteins.

TcMTAP has been characterized as a dimer [21] and our previous work [14] showed that rTcMTAP subunits association is probably kept by weak interactions. Besides that, data from dynamic light scattering corroborate that rTcMTAP associate into oligomeric species (data not shown). It has been shown that some proteins assemble into dimers through weak interations, but still present a two-state denaturation. In this case, the thermodynamic stability comes from the stabilization of each monomer [22,23]. Sigmoidal curves, characteristic of cooperative unfolding transitions, were obtained for rTcMTAP under chemical and thermal denaturation conditions, accompanied by CD and fluorescence spectroscopy. These data indicate that the dimer structural arrangement of rTcMTAP denatures in a two-state pathway because no alteration on spectra prior the transition was detected.

Thus, the analysis of denaturation curves in this study was based on the assumption that only the folded and the unfolded forms of rTcMTAP exist at equilibrium. This assumption is based on that dimer dissociation leads to the formation of an intrinsically unstable species, hence folded monomeric species will not significantly populate at equilibrium. Although dimeric proteins have additional modes of quaternary structure stabilization, many still follow a two-state transition [9,24].

The values of thermodynamic parameters indicate that rTcMTAP exhibits considerable stability at acid and neutral pH, but not at basic pH. This correlates well to

the activity of rTcMTAP in a broad pH range, expect at pH 8.6 or higher [14]. The lower activity at basic pH might be correlated the altered transition curves observed at these conditions. The CD signal fluctuation observed prior to 70 °C at over-7.4 pH is probably due to ionization effect altering the intramolecular and intermolecular interactions associated with the catalytic sites.

A possible evolutionary explanation to rTcMTAP thermal resistance is the primary sequence identity with MTAPs from thermophilic organisms [14]. rTcMTAP highest stability in the absence of stabilizers CTAB and sorbitol was observed at pH 7.4 with phosphate. This result indicates that the binding of this substrate raises the conformational stability of the enzyme, thus reducing its susceptibility to thermal denaturation. Phosphate induced similar protection in MTAP from *Sulfolobus solfataricus* and *Pyrococcus furiosus* [25,26]. In contrast, the lowest $\Delta G^{25}$ was obtained at pH 4.0 which may reflect that lower pH cause disruption of critical interactions upon protonation, thus decreasing protein stability [27].

Sorbitol, a polyol, probably increased rTcMTAP stability, but the thermodynamic parameters were impossible to calculate because the slopes of thermal denaturarion did not exhibit an evident unfolding pattern. Although sorbitol increased the CD signal, it did not alter the shape of the spectra which we suppose to reflect alterations in protein compactness or oligomerization state. Some sugars and polyols have been found to raise protein's denaturation temperature by as much as 15 °C [28]. The most common explanation to sorbitol's protective effect is that this osmolyte raises the surface tension of the medium, which leads to a preferential hydration of the protein, resulting in a decreased hydrogen-bond rupturing capacity of the medium [29-31]. In contrast, CTAB, a cationic surfactant, increased protein's $\Delta G^{25}$ but did not avoid rTcMTAP thermal denaturation. The high stability at pH 6.0 was mostly entropy-driven, reflecting an improved organization of environment molecules with rTcMTAP. In opposite, the reduced stability induced by CTAB at pH 7.4 was enthalpy-driven, indicationg rupture of enzyme intramolecular bonds. CTAB at concentration below its critical micelle concentration (c.m.c = 1.1 mM) seems to interact with apolar parts through introducing its hydrophobic chain inside hydrophobic cavities of the protein [32]. We observed that CTAB at pH 7.4 has a negative influence on enzyme stability, probably because it perturbs, what seems to be, the best configuration of non-covalent interactions that rTcMTAP establishes. CTAB also induced both increasing and red-shifting of rTcMTAP fluorescence indicating that

Trp residues are more exposed to polar environment. The increased intensity of fluorescence suggests that the exposure of Trp167 led to disruption of the internal quenching effect of Arg168 in the native conformation, which is a known quenching circumstance [33].

The enzyme exhibited remarkable resistance to chemical denaturation keeping folded in concentrations as high as 3.6 M of Gnd-HCl and 8 M. The decreased intensity of fluorescence prior to 3.6 M Gnd-HCl and in the urea assay is the quenching effect caused by higher concentration of the denaturant. Comparing the $\Delta G_u(H_2O)$ from CD data (35.5 Kcal/mol) and the $\Delta G^{25}$ from thermal denaturation we observe a good concordance between the results from CD indicating that this technique is likely to reflect the full unfolding process of rTcMTAP. Since results from thermal and chemical denaturation were coincident corroborates the assumption that rTcMTAP unfolds in a two-way fashion. To confirm this supposition we suggest that different approaches should be used, e. g. fluorescence and CD in different protein concentrations and conditions, nuclear magnetic resonance, in addition to activity measurements and differential scanning calorimetry (DSC). In addition, Neet [9], suggested a relationship between the size of the dimer and the protein structure stabilization, since a linear correlation was observed between the number of amino acid residues ($N$) in the monomer and the value of $\Delta G_u^{H2O} = 8.8 + 0.08N$ kcal/mol. The formula-predicted $\Delta G_u(H_2O)$ is 35.2 kcal/mol, a close estimative to that experimentally obtained, again corroborating the results from CD data.

In this work we characterized thermodynamically the recombinant methylthioadenosine phosphorylase of *T. cruzi*. The enzyme exhibits remarkable chemical and thermal resistance; the latter was increased in presence of stabilizer sorbitol and CTAB. The highest stability was achieved at pH 7.4 in the presence of phosphate, corroborating the protection role played by this substrate. However, the tested additives seem to alter the enzyme's secondary and tertiary structure. We also observed that rTcMTAP unfolds in a two-state model.

## MATERIALS AND METHODS

### Recombinant protein production and purification

Recombinant TcMTAP was heterologously produced in *E. coli* BL21-DE3 strain and purified as described [14]. Enzyme stock used in our experiments was not more than 1 week old. The protein purity was determined by SDS-PAGE and concentration was monitored by measuring the absorbance at 280 nm using the extinction coefficient of 21980 $M^{-1}.cm^{-1}$, calculated using the Protparam tool (http://www.expasy.org/tools/protparam.html).

**Circular dichroism spectroscopy**

Circular dichroism measurements were carried out on a JASCO J-810 spectropolarimeter equipped with a Peltier-type temperature controller and a thermostated cell holder interfaced with a thermostatic bath. Far-UV spectra (185 to 260 nm) were recorded in 0.1-cm pathlength quartz cells at a protein concentration of 0.1 mg/mL (3.0 μM) in 2.5 mM buffers: Na-acetate pH 4.0, Sodium-acetate pH 5.2; bis-Tris pH 6.0, Potassium-phosphate pH 7.4, or Tris-HCl pH 9.0. Spectra from pH 6.0 and 7.4 in the presence of CTAB (0.1, 0.5 and 1.0 mM) or sorbitol (0.5 and 1.0 M) were also collected. To determine the thermodynamic parameters we performed thermal denaturation at the above pH and also at Tris-HCl pH 7.4, 8.0 and 8.6. The spectra presented in this work represent the average of four accumulated consecutive scans. Thermal denaturation assays were performed by increasing the temperature from 20 to 95 °C, allowing the temperature stabilization for 5 min before recording each spectrum. The data were corrected for the baseline contribution of the buffer and the observed ellipticities at 208 nm were recorded. The ellipticities were converted into the mean residue ellipticities [θ] based on the mean molecular mass residue of rTcMTAP. Data were analyzed by assuming a two-state transition considering the changes in the [θ]. The overall unfolding reaction starts with the folded dimer ($A_2$) and end with two unfolded monomers (2U). The overall reaction may be described by the following:

$$A_2 \overset{K_U}{\leftrightarrow} 2U$$

Where, $K_U = [U]^2 / [A_2] = 4 f_d^2 P_t / (1- f_d)$ and $\Delta G_u = -RT \text{ Ln } K_u$

$K_u$ is the dissociation constant, $f_d$ is the molar fraction of denatured protein, $P_t$ is the total protein molar concentration (monomers unit). Evaluation of $K_u$ and extrapolation to standard conditions to obtain Gibbs free energy ($\Delta G_u$) can be performed as for monomeric

proteins. Thermodynamic parameters derived from the transition curves were calculated by linear extrapolation method (LEM).

Secondary structure content was estimated from far-UV CD spectra using the SELCON3, CDSSTR and CONTINLL software, from CDPro suite (http://lamar.colostate.edu/~sreeram/CDPro). It was employed the reference set of 50 proteins, SMP50. Showed contents are an average of the three programs results.

**Isothermal Guanidine hydrochloride-induced Denaturation**

Equilibrium unfolding as function of guanidine hydrochloride (Gnd-HCl) concentration was monitored by fluorescence spectroscopy and circular dichroism. Each sample of rTcMTAP, 0.1 mg/ mL in 10 mM potassium phosphate buffer pH 7.4, was incubated in the presence of 0-7 M Gnd-HCl or 0-8 M urea, both at 0.2 M intervals, and equilibrated for 24 h prior measurements. Fluorescence measurements were carried on an Aminco Bowman Series 2 (SLM Aminco) luminescence spectrometer using a 1 × 1 cm path length cuvette with water-jacketed cell previously stabilized at 25 °C in all experiments. Excitation wavelength was at 280 nm and the emission was recorded from 310 to 420 nm with 1 nm increment. The CD data were collected by the same apparatus used in circular dichroism assay. Each condition spectra were corrected subtracting the signal of the buffer different denaturant concentrations.

**REFERENCES**


[1] Avila MA, Garcia-Trevijano ER, Lu SC, Corrales FJ & Mato JM (2004). Methylthioadenosine. *Int J Biochem Cell Biol* **36**, 2125-2130.

[2] Pollock PM, Pearson JV & Hayward NK (1996). Compilation of somatic mutations of the CDKN2 gene in human cancers: non-random distribution of base substitutions. *Genes Chromosomes Cancer* **15**, 77-88.

[3] Smith-Sorensen B & Hovig E (1996). CDKN2A (p16INK4A) somatic and germline mutations. *Hum Mutat* **7**, 294-303.

[4] Ghoda LY, Savarese TM, Northup CH, Parks RE, Jr., Garofalo J, Katz L, Ellenbogen BB & Bacchi CJ (1988). Substrate specificities of 5'-deoxy-5'-

methylthioadenosine phosphorylase from Trypanosoma brucei brucei and mammalian cells. *Mol Biochem Parasitol* **27**, 109-118.

[5] Bacchi CJ, Sufrin JR, Nathan HC, Spiess AJ, Hannan T, Garofalo J, Alecia K, Katz L & Yarlett N (1991). 5'-Alkyl-substituted analogs of 5'-methylthioadenosine as trypanocides. *Antimicrob Agents Chemother* **35**, 1315-1320.

[6] Bacchi CJ, Sanabria K, Spiess AJ, Vargas M, Marasco CJ, Jr., Jimenez LM, Goldberg B & Sufrin JR (1997). In vivo efficacies of 5'-methylthioadenosine analogs as trypanocides. *Antimicrob Agents Chemother* **41**, 2108-2112.

[7] Sacchetta P, Aceto A, Bucciarelli T, Dragani B, Santarone S, Allocati N & Di Ilio C (1993). Multiphasic denaturation of glutathione transferase B1-1 by guanidinium chloride. Role of the dimeric structure on the flexibility of the active site. *Eur J Biochem* **215**, 741-745.

[8] Brandau DT, Jones LS, Wiethoff CM, Rexroad J & Middaugh CR (2003). Thermal stability of vaccines. *J Pharm Sci* **92**, 218-231.

[9] Neet KE & Timm DE (1994). Conformational stability of dimeric proteins: quantitative studies by equilibrium denaturation. *Protein Sci* **3**, 2167-2174.

[10] Holdgate GA & Ward WH (2005). Measurements of binding thermodynamics in drug discovery. *Drug Discov Today* **10**, 1543-1550.

[11] Urbina JA (2001). Specific treatment of Chagas disease: current status and new developments. *Curr Opin Infect Dis* **14**, 733-741.

[12] Bacchi CJ, Goldberg B, Rattendi D, Gorrell TE, Spiess AJ & Sufrin JR (1999). Metabolic effects of a methylthioadenosine phosphorylase substrate analog on African trypanosomes. *Biochem Pharmacol* **57**, 89-96.

[13] Berens RL KE, Marr JJ (1995) in: *Biochemistry and molecular biology of parasites.*, pp. 89-117 (M, M.J.a.M., Ed.) Academic Press, London.

[14] Neves D, Bastos ID, de Lima MM, Restrepo-Cadavid G, Teixeira ARL, Medrano FJ, Grellier P, Schrevel J, Campbell DA & Santana JM (2006). The Methylthioadenosine phosphorylase of *Trypanosoma cruzi* is mesophilic and displays broad substrate specificity.

[15] Wielgus-Kutrowska B, Bzowska A, Tebbe J, Koellner G & Shugar D (2002). Purine nucleoside phosphorylase from Cellulomonas sp.: physicochemical properties and binding of substrates determined by ligand-dependent enhancement

of enzyme intrinsic fluorescence, and by protective effects of ligands on thermal inactivation of the enzyme. *Biochim Biophys Acta* **1597**, 320-334.

[16] Bolotina IA (1987). [Secondary structure of proteins from circular dichroism spectra. V. Secondary structure of proteins in a "molten globule" state]. *Mol Biol (Mosk)* **21**, 1625-1635.

[17] Santoro MM & Bolen DW (1992). A test of the linear extrapolation of unfolding free energy changes over an extended denaturant concentration range. *Biochemistry* **31**, 4901-4907.

[18] Teale FW (1960). The ultraviolet fluorescence of proteins in neutral solution. *Biochem J* **76**, 381-388.

[19] Timm DE, de Haseth PL & Neet KE (1994). Comparative equilibrium denaturation studies of the neurotrophins: nerve growth factor, brain-derived neurotrophic factor, neurotrophin 3, and neurotrophin 4/5. *Biochemistry* **33**, 4667-4676.

[20] Wallace LA, Sluis-Cremer N & Dirr HW (1998). Equilibrium and kinetic unfolding properties of dimeric human glutathione transferase A1-1. *Biochemistry* **37**, 5320-5328.

[21] Miller RL, Sabourin CL & Krenitsky TA (1987). Trypanosoma cruzi adenine nucleoside phosphorylase. Purification and substrate specificity. *Biochem Pharmacol* **36**, 553-560.

[22] Cunningham BC, Mulkerrin MG & Wells JA (1991). Dimerization of human growth hormone by zinc. *Science* **253**, 545-548.

[23] Pace NC & Tanford C (1968). Thermodynamics of the unfolding of beta-lactoglobulin A in aqueous urea solutions between 5 and 55 degrees. *Biochemistry* **7**, 198-208.

[24] Ahmad N, Srinivas VR, Reddy GB & Surolia A (1998). Thermodynamic characterization of the conformational stability of the homodimeric protein, pea lectin. *Biochemistry* **37**, 16765-16772.

[25] Cacciapuoti G, Forte S, Moretti MA, Brio A, Zappia V & Porcelli M (2005). A novel hyperthermostable 5'-deoxy-5'-methylthioadenosine phosphorylase from the archaeon Sulfolobus solfataricus. *Febs J* **272**, 1886-1899.

[26] Cacciapuoti G, Bertoldo C, Brio A, Zappia V & Porcelli M (2003). Purification and characterization of 5'-methylthioadenosine phosphorylase from the

hyperthermophilic archaeon Pyrococcus furiosus: substrate specificity and primary structure analysis. *Extremophiles* **7**, 159-168.

[27] Hughson FM & Baldwin RL (1989). Use of site-directed mutagenesis to destabilize native apomyoglobin relative to folding intermediates. *Biochemistry* **28**, 4415-4422.

[28] Back JF, Oakenfull D & Smith MB (1979). Increased thermal stability of proteins in the presence of sugars and polyols. *Biochemistry* **18**, 5191-5196.

[29] Gerlsma S (1968). Reversible denaturation of ribonuclease in aqueous solutions as influenced by polyhydric alcohols and some other additives. *The Journal of biological chemistry* **243**, 957-961.

[30] Gerlsma SY (1970). The effects of polyhydric and monohydric alcohols on the heat induced reversible denaturation of chymotrypsinogen A. *Eur J Biochem* **14**, 150-153.

[31] Tiwari A & Bhat R (2006). Stabilization of yeast hexokinase A by polyol osmolytes: Correlation with the physicochemical properties of aqueous solutions. *Biophys Chem*.

[32] Liu W, Guo X & Guo R (2005). The interaction of hemoglobin with hexadecyltrimethylammonium bromide. *Int J Biol Macromol* **37**, 232-238.

[33] Permyakov E (1993) CRC Press, Boca Raton, FL, USA.

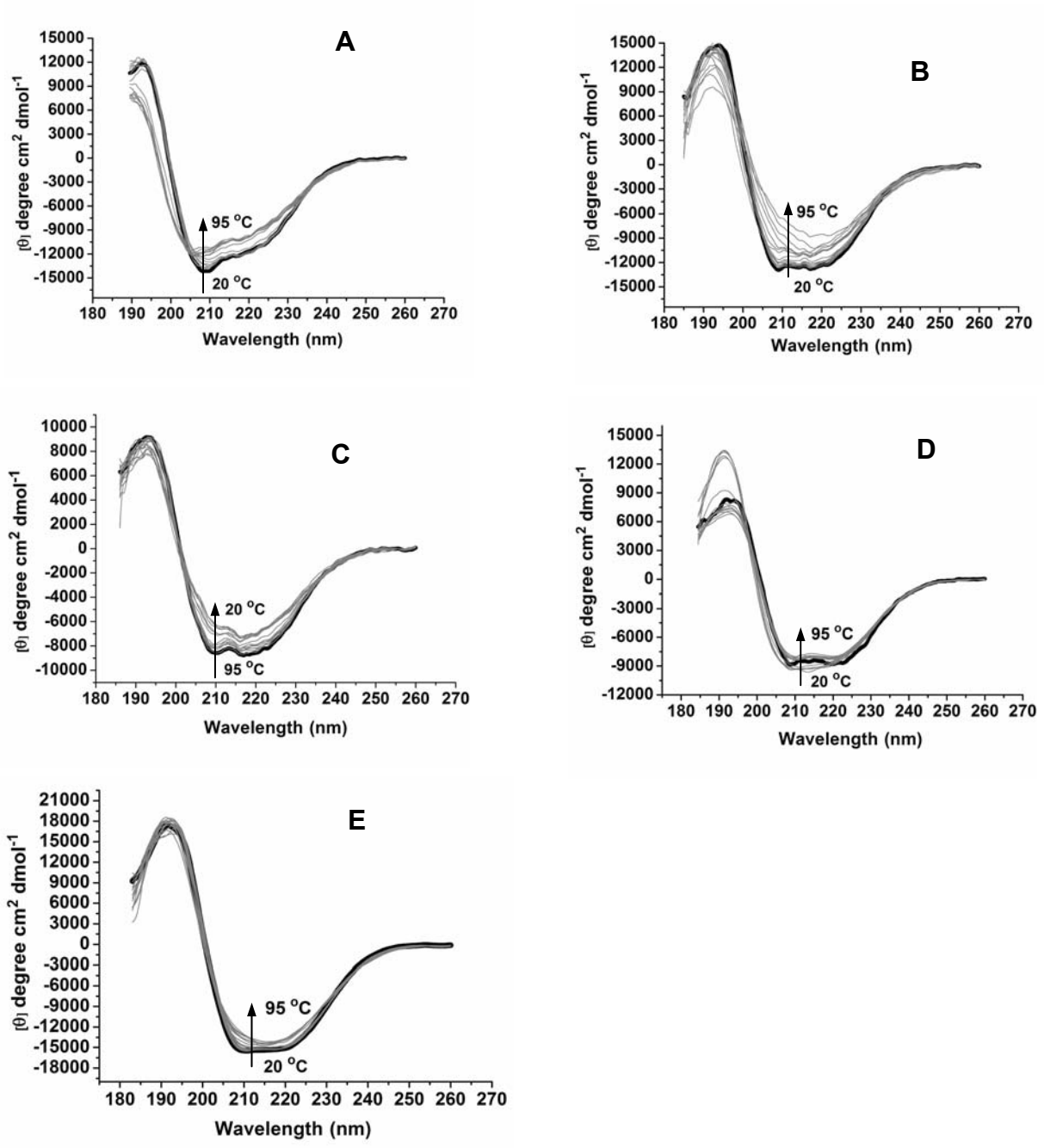


**Fig. 1**. **rTcMTAP spectra exhibit less variation at higher pH.** Far-UV CD spectra at pH: (A) 4.0; (B) 5.2; (C) 6.0; (D) 7.4; and (E) 9.0 were recorded during thermal denaturation. The bolded spectrum corresponds to 20 °C data.

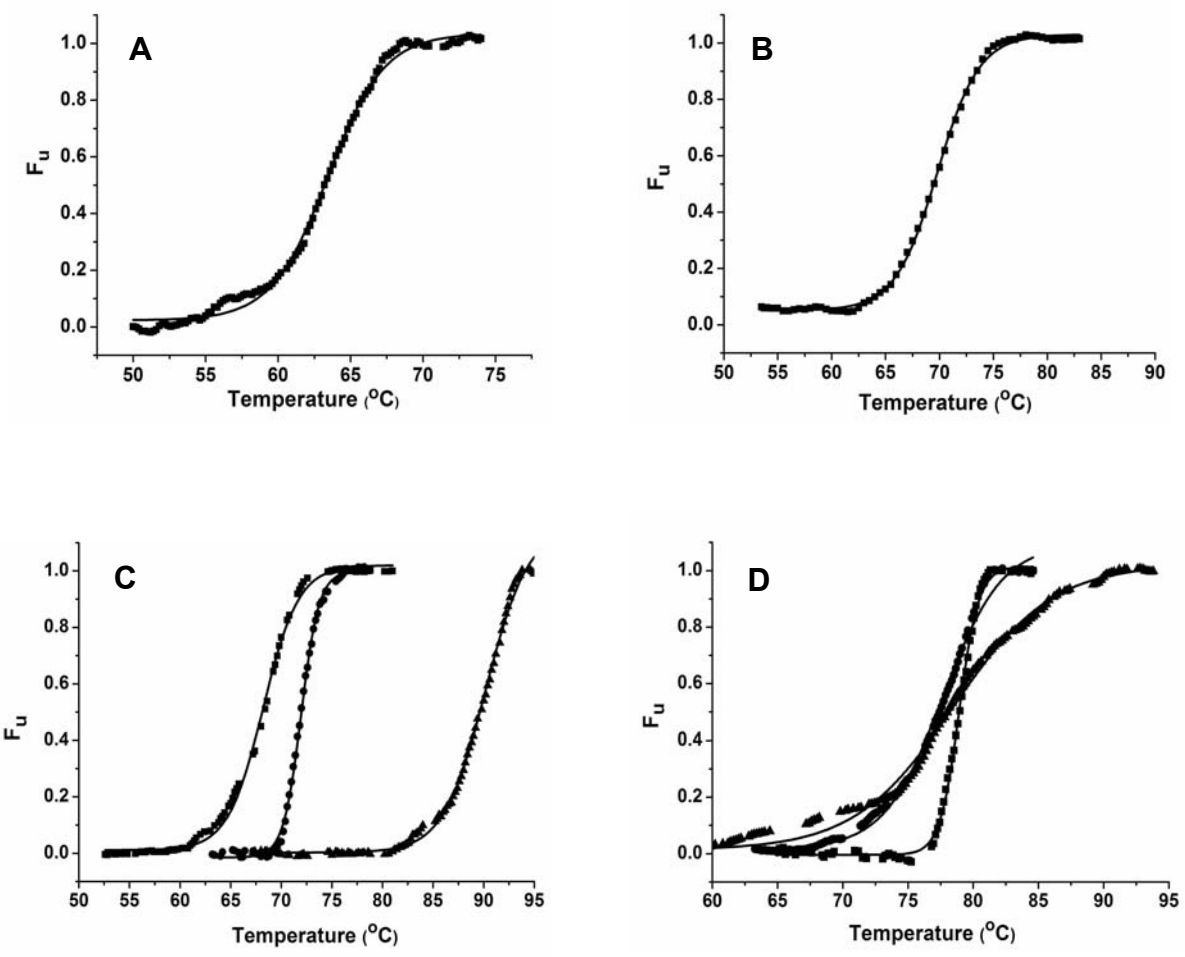


**Fig. 2. rTcMTAP resistance to thermal denaturation varies with pH and in the presence of CTAB.** Fitted unfolding curves from thermal denaturation data performed at pH: (A) pH 4.0 (B) pH 5.2 (C) pH 6.0 (D) pH 7.4 and in the (■) absence or in the presence of (●) 0.1 mM and (▲) 0.5 mM CTAB. The unfolding process was followed by far-UV CD at 208 nm.

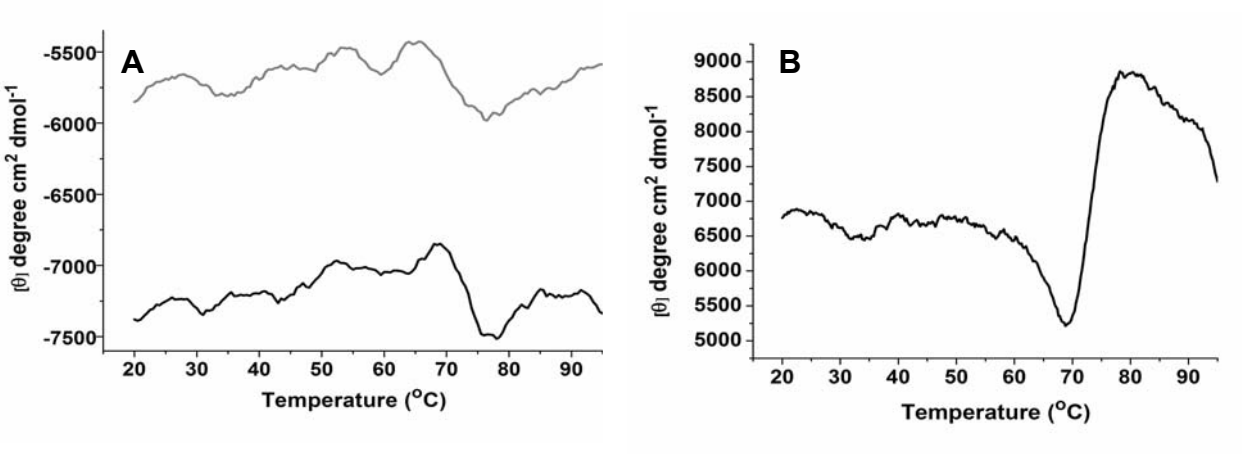


**Fig. 3. rTcMTAP transition slope has altered pattern at high pH.** Thermal denaturation process exhibited a non-two-state denaturation at pH: (A) 8.0 (gray); 8.6 (black) and (B) 9.0.

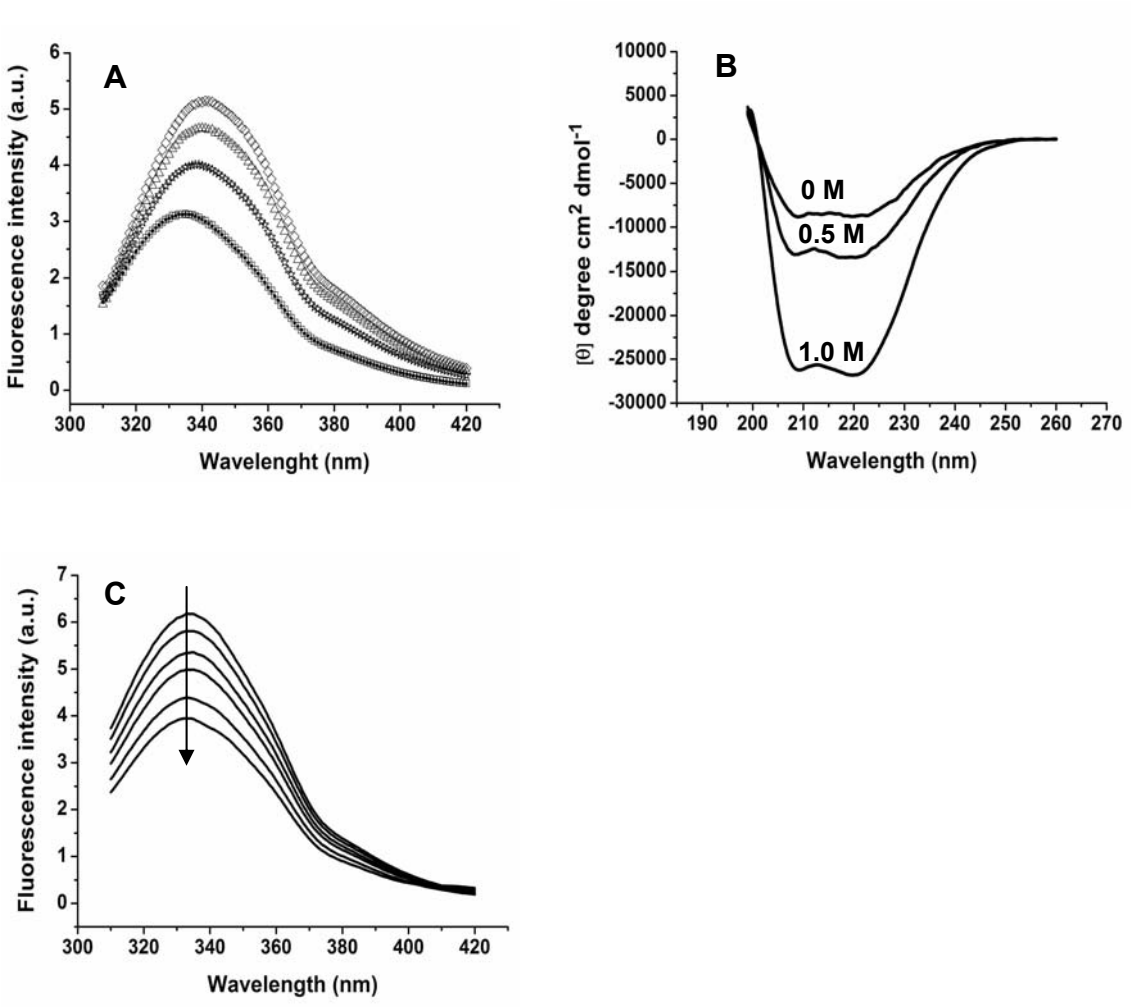


**Fig. 4. Stabilizers affect rTcMTAP fluorescence and CD spectra.** (A) Fluorescence spectra in the (●) absence or in the presence of (□) 0.1 mM, (ψ) 0.5 mM, (△) 1.0 mM and (◊) 2.0 mM CTAB at pH 7.4. (B) CD spectra in the presence of Sorbitol (0 – 1 M). (C) Fluorescence spectra in the presence of Sorbitol: 0 – 1.1 M (bottom).

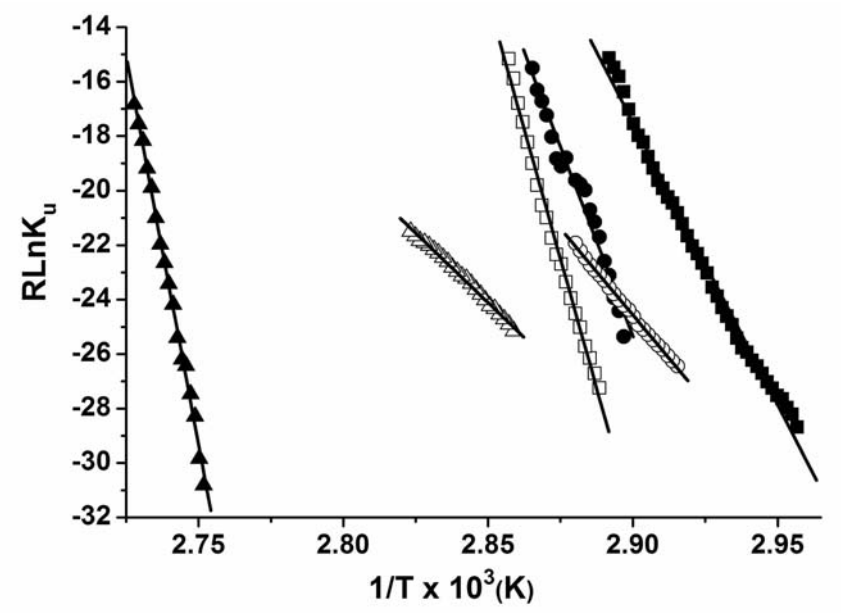


**Fig. 5. Comparison of vant't Hoff plot at different pH in absence and presence of CTAB.** Data from thermal denaturation at pH 6.0 - filled symbols or pH 7.4 - open symbols in the (!) absence or presence of (,) 0.1 mM, (7) 0.5 mM CTAB were fitted to van't Hoff expression.

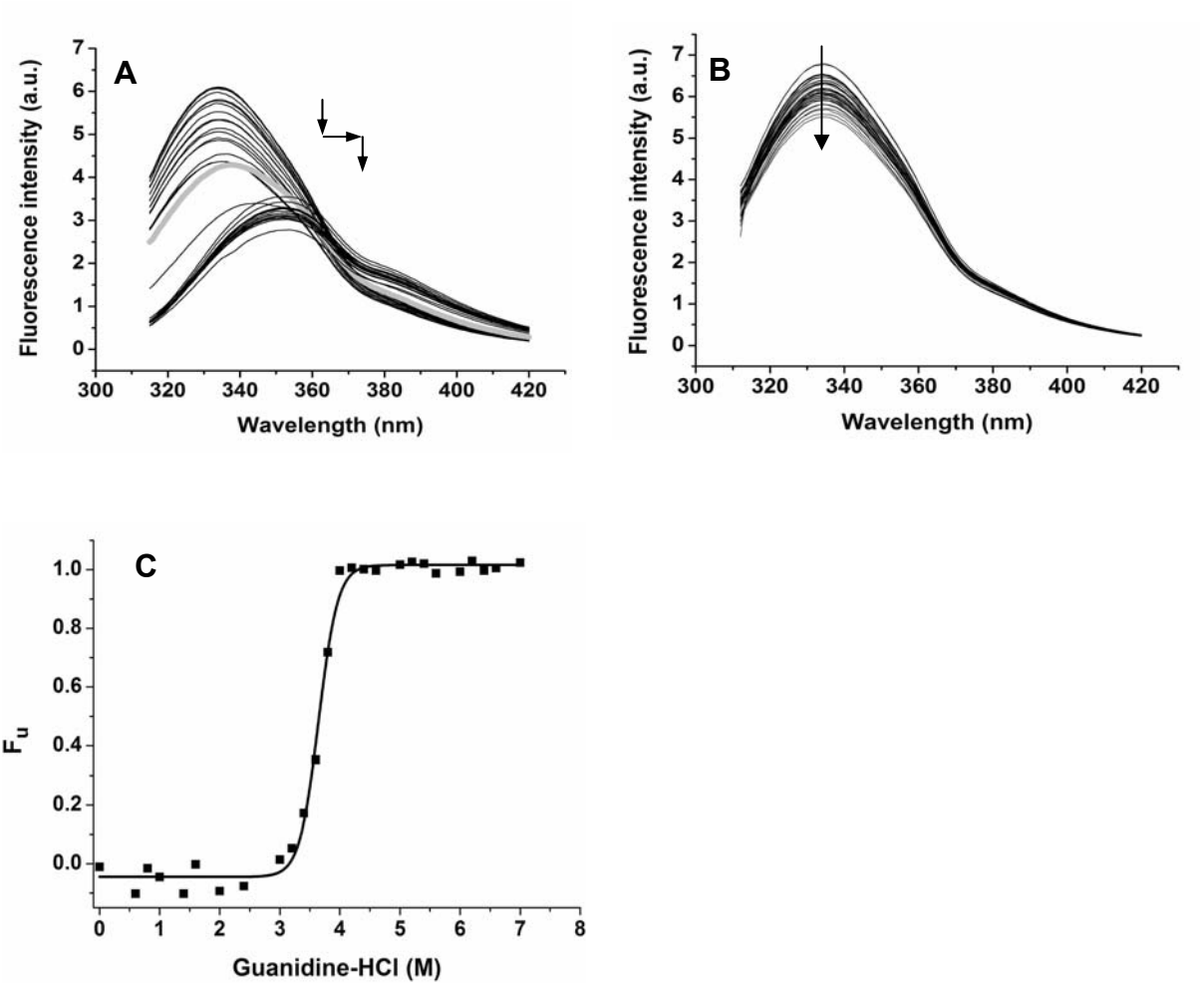


**Fig. 6. rTcMTAP keep folded until 3.6 M Gnd-HCl or 8 M urea.** Fluorescence emission spectra of rTcMTAP in presence of: (A) Gnd-HCl (0 – 7 M), gray spectrum represents 3.8 M. (B) urea (0 – 8 M). (C) Fitted Gnd-HCl-induced unfolding curve followed by CD. The arrows indicate the maximum wavelength path throughout the assay.

**Table 1. Thermal denaturation has discrete effect over rTcMTAP secondary structure content.** Each structure content is the average of CDSSTR, CONTINLL and SELCON3 programs estimation for every condition.

| pH | Temperature (°C) | α-Helix (%) | β-Sheet (%) | β-Turn (%) | Unordered (%) | Total (%) |
|---|---|---|---|---|---|---|
| 4.0 | 20 | 37 | 14 | 22 | 28 | 100 |
| | 95 | 21 | 24 | 23 | 32 | 100 |
| 5.0 | 20 | 37 | 15 | 20 | 28 | 100 |
| | 95 | 22 | 29 | 21 | 28 | 99 |
| 6.0 | 20 | 24 | 25 | 21 | 29 | 99 |
| | 95 | 13 | 35 | 23 | 29 | 100 |
| 7.4 | 20 | 26 | 24 | 21 | 29 | 99 |
| | 95 | 24 | 24 | 22 | 28 | 99 |
| 9.0 | 20 | 40 | 13 | 20 | 27 | 100 |
| | 95 | 36 | 17 | 20 | 27 | 100 |

**Table 2. CTAB and phosphate influences rTcMTAPthermodynamic parameters.** Thermodynamic parameters, melting temperature ($T_m$), thermal stability at 25 °C ($\Delta G^{25}$), enthalpy ($\Delta H_u$) and entropy ($\Delta S_u$) at unfolding were calculated at different pH in the absence or presence of CTAB.

| pH | $T_m$ (ºC) | $\Delta G^{25}$ (kcal/ mol) | $\Delta H_u$ (kcal/ mol) | $\Delta S_u$ (cal/ mol) |
|---|---|---|---|---|
| 4.0 | 79.8 | 27.7 | 180 ± 2 | 512 ± 6 |
| 5.2 | 80.5 | 28.9 | 167 ± 2 | 465 ± 5 |
| 6.0 | 82.8 | 32.5 | 200 ± 3 | 564 ± 7 |
| 6.0 + 0.1 mM CTAB | 80.6 | 47.1 | 290 ± 1 | 816 ± 36 |
| 6.0 + 0.5 mM CTAB | 97.6 | 112.6 | 575 ± 8 | 1553 ± 23 |
| 7.4 | 82.0 | 36.0 | 213 ± 3 | 590 ± 7 |
| 7.4 + Phosphate | 81.9 | 61.2 | 380 ± 6 | 1071 ± 16 |
| 7.4 + 0.1 mM CTAB | 98.6 | 25.3 | 132 ± 2 | 360 ± 5 |
| 7.4 + 0.5 mM CTAB | 118.7 | 23.5 | 108 ± 2 | 284 ± 6 |

# Livros Grátis

( http://www.livrosgratis.com.br )

Milhares de Livros para Download:

Baixar livros de Literatura
Baixar livros de Literatura de Cordel
Baixar livros de Literatura Infantil
Baixar livros de Matemática
Baixar livros de Medicina
Baixar livros de Medicina Veterinária
Baixar livros de Meio Ambiente
Baixar livros de Meteorologia
Baixar Monografias e TCC
Baixar livros Multidisciplinar
Baixar livros de Música
Baixar livros de Psicologia
Baixar livros de Química
Baixar livros de Saúde Coletiva
Baixar livros de Serviço Social
Baixar livros de Sociologia
Baixar livros de Teologia
Baixar livros de Trabalho
Baixar livros de Turismo