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DEPARTAMENTO DE BIOQUÍMICA

MAPEAMENTO DO PADRÃO DE EXPRESSÃO TECIDUAL DOS GENES RELACIONADOS À ADENOSINA DEAMINASE E CARACTERIZAÇÃO CINÉTICA DA ATIVIDADE DE DESAMINAÇÃO DA ADENOSINA EM CÉREBRO DE ZEBRAFISH (*Danio rerio*)

DENIS BROOCK ROSEMBERG

Orientador: Prof. Dr. Diogo Onofre Souza

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Sei que meu trabalho é uma gota no oceano,

mas sem ele, o oceano seria menor.

Madre Teresa de Calcutá

A ousadia é, depois da prudência, uma

condição especial da nossa felicidade.

Arthur Schopenhauer

A palavra do bioquímico pode não ser a última na descrição da vida,

mas sem sua ajuda, a última palavra nunca será dita.

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PARTE I

I.1. Resumo

O zebrafish é um modelo experimental consolidado em diversas áreas, tais como genética e neurociências. Estudos têm demonstrado que muitos genes deste peixe são evolutivamente conservados e similares aos de mamíferos, incluindo a espécie humana. Com relação ao sistema purinérgico, já foi demonstrado que o zebrafish apresenta diferentes membros da família das NTPDases (nucleosídeo trifosfato difosfoidrolases) e uma ecto-5'-nucleotidase, capazes de clivar o ATP até adenosina, que atua através de purinoreceptores P1.

A adenosina deaminase (ADA) é responsável pela clivagem da adenosina à inosina. Dois membros da família da ADA, conhecidos como ADA1 e ADA2, foram descritos e evidências recentes demonstraram a existência de um outro grupo similar de proteína, denominado ADAL (adenosina deaminase “like”). Portanto, no primeiro capítulo desta Dissertação, foram identificados distintos genes relacionados à ADA (*ADA1*, *ADAL* e dois genes ortólogos da *ADA2*) através de uma análise filogenética. Primers específicos para cada membro da ADA foram desenhados, experimentos otimizados de RT-PCR foram conduzidos e a quantidade relativa de transcritos determinada em diversos tecidos. Os resultados demonstraram que os genes da *ADA1*, *ADAL*, *ADA2-1* e *ADA2-2* podem ser diferentemente expressos nos tecidos de zebrafish. Além disso, a estratégia adotada também permitiu a identificação de uma isoforma truncada de *ADA2-1* de splicing alternativo (*ADA2-1/T*), a qual foi expressa em diferentes intensidades nos tecidos analisados.

Considerando que distintos membros da adenosina deaminase foram identificados, o segundo capítulo teve por objetivo caracterizar a atividade de desaminação de adenosina em frações solúvel e de membrana do cérebro de zebrafish. A atividade enzimática foi determinada pelo ensaio colorimétrico da amônia liberada. Foi verificado que em ambas as frações celulares estudadas a atividade de desaminação da adenosina foi linear quando utilizada uma concentração final de substrato de 1,5 mM. A curva de proteína, após incubação a 37ºC por 75 min (pH 7,0) e 120 min (pH 5,0) para as frações solúvel e de membrana, respectivamente, foi linear quando utilizada uma quantidade de proteína na faixa de 5–20 µg. A adição de 5 mM de Zn^{2+} promoveu uma queda significativa na desaminação de adenosina em membranas, a qual foi prevenida com 5 mM de EDTA. Utilizando adenosina como substrato, o K_M aparente para ambas as frações celulares foi de aproximadamente 0,2 mM, enquanto que o V_{max} foi de 12,3 $\pm$ 0,73 e 17,5 $\pm$ 0,51 (média $\pm$ EP) nmol $NH_3.min^{-1}.mg^{-1}$ de proteína para as frações solúvel e de membrana, respectivamente. Os resultados também demonstraram uma preferência para a desaminação de nucleosídeos da adenina em relação aos da guanina. Além disso, a incubação com 0,1 mM de EHNA (hidrocloreto de eritro-9-(2-hidróxi-3-nonil) adenina), um inibidor clássico da ADA1, promoveu uma inibição significativa da atividade enzimática em ambas as frações celulares.

Estes achados sugerem que a existência de diferentes genes associados à ADA, bem como seus distintos padrões de expressão podem contribuir para a atividade de desaminação de adenosina em zebrafish.

I.2. Abstract

Zebrafish (*Danio rerio*) is a consolidated experimental model in several areas, such as genetics and neuroscience. Studies have shown that many genes of this fish are evolutionary conserved and that share similarities to mammals genes, including humans. In relation to the purinergic system, it was demonstrated that zebrafish presents distinct members of the NTPDase (nucleoside triphosphate diphosphohydrolase) family and an ecto-5'-nucleotidase, able to cleave ATP to adenosine, which acts via P1 purinoreceptors.

Adenosine deaminase (ADA) is responsible for cleaving the adenosine to inosine. Two members of ADA family, known as ADA1 and ADA2, were described and recent evidence demonstrated the existence of another similar protein group named ADAL (adenosine deaminase "like"). Therefore, in the first chapter of this Dissertation, distinct ADA-related genes were identified (*ADA1*, *ADAL* e two *ADA2* orthologous genes) by a phylogenetic analysis. Specific primers for each ADA members were designed, optimized semi-quantitative RT-PCR experiments were conducted and the relative amount of transcripts was determined in several tissues. The results demonstrated that *ADA1*, *ADAL*, *ADA2-1* e *ADA2-2* genes may be expressed differently in zebrafish tissues. In addition, the strategy adopted also allowed the identification of a truncated alternative splice isoform of *ADA2-1*, which was expressed in the analyzed tissues with different intensities.

Considering that distinct adenosine deaminase members were identified, the objective of the second chapter was to characterize the adenosine deaminase activity in soluble and membrane fractions of zebrafish brain. The enzyme activity was measured by the colorimetric assay from the ammonia released. It was verified that in both cellular fractions, the adenosine deaminase activity was linear when it was used a final substrate concentration of 1.5 mM. The protein curve was linear using an amount of 5–20 µg of protein in the incubation at 37ºC for 75 min (pH 7.0) and 120 min (pH 5.0) for soluble and membrane fractions, respectively.

The addition of 5 mM Zn^{2+} promoted a significant decrease on adenosine deamination in membranes, which was prevented by 5 mM EDTA. Using adenosine as substrate, the apparent K_M for both cellular fractions was around of 0.2 mM, whereas the calculated V_{max} were 12.3 $\pm$ 0.73 and 17.5 $\pm$ 0.51 (mean $\pm$ SEM) nmol $NH_3.min^{-1}.mg^{-1}$ of protein for the soluble and membrane fractions, respectively. The results also demonstrated a preference for the deamination of adenine nucleosides in relation to guanine derivates. In addition, the incubation with 0.1 mM EHNA (erythro-9-(2-hydroxy-3-nonyl)-adenine, a classical inhibitor of ADA1, promoted a significant inhibition on the enzyme activity in both cellular fractions.

These findings suggest that the existence of different ADA-related genes and their distinct expression patterns may contribute for the adenosine deamination activity in zebrafish.

I.3. Lista de abreviaturas

ADA – adenosina deaminase

ADA1 – adenosina deaminase 1

ADA2 – adenosina deaminase 2

ADA2-1 – parálogo 1 da adenosina deaminase 2

ADA2-1/T – parálogo 1 da adenosina deaminase 2 truncada

ADA2-2 – parálogo 2 da adenosina deaminase 2

ADAL – adenosina deaminase “like”

ADE – adenina deaminase

ADGF – “adenosine deaminase-related growth factor” (fator de crescimento relacionado à ADA)

ADP – adenosina 5’-difosfato

ADO – adenosina

AK – adenosina cinase

AMP – adenosina 5’-monofosfato

AMPD – adenosina 5’-monofosfato deaminase

ATP – adenosina 5’-trifosfato

cDNA – ácido desoxirribonucléico complementar

CD26 – marcador de superfície correspondente à dipeptidil peptidase IV

CECR1 – “cat-eye syndrom critical region 1” (região crítica 1 da síndrome do olho de gato)

2’-D-ADO – 2’-desoxiadenosina

2’-D-INO – 2’-desoxinosina

DPPIV – dipeptidil peptidase IV

Ecto-ADA – ecto adenosina deaminase

EHNA – hidrocloreto de eritro-9-(2-hidroxi-3-nonil) adenina

ESTs – “expressed sequence tags” (genes expressos)

GABA – ácido γ-aminobutírico

INO – inosina

K_M – constante de Michaelis

mRNA – ácido ribonucléico mensageiro

NTPDase – nucleosídeo trifoafato difosfoidrolase

PCR – “polymerase chain reaction” (reação em cadeia da polimerase)

RNA – ácido ribonucléico

RT-PCR – “reverse transcription-polymerase chain reaction” (reação em cadeia da polimerase com transcrição reversa)

SIDSC – síndrome da imunodeficiência severa combinada

SNC – sistema nervoso central

TCDD – 2,3,7,8-tetraclorodibenzeno-*p*-dioxina

ZFIN – “zebrafish international network” (rede internacional de dados do zebrafish)

I.4. Introdução

I.4.1. Zebrafish (*Danio rerio*)

O zebrafish (*Danio rerio*), também conhecido por peixe-zebra ou "paulistinha" pelos aquariofilistas é um pequeno teleósteo da família Cyprinidae. O pioneiro a estudar esta espécie foi George Streisinger, o qual, no final da década de 60, utilizou técnicas de análise mutacional para avaliar o desenvolvimento embrionário do zebrafish (GRUNWALD & EISEN, 2002). A descrição inicial do uso do zebrafish em estudos científicos envolvendo biologia do desenvolvimento possibilitou um grande avanço no conhecimento da embriogênese e do ciclo de vida dos vertebrados. Isto se deve a diversas vantagens que este animal apresenta, tais como a presença de ovos translúcidos, a grande prole e o curto ciclo de desenvolvimento, cuja fase do ovo ao adulto leva aproximadamente dois meses (LELE & KRONE, 1996). A embriogênese possui uma duração aproximada de 24 horas e a organogênese apresenta seu estágio final no quinto dia de desenvolvimento (DAHM & GEISLER, 2006). A transparência do córion e a translucência do embrião e das fases larvais iniciais permite uma fácil visualização dos processos internos, como a formação e função de órgãos em um animal vivo (DAHM & GEISLER, 2006). Além disto, esta característica dos embriões de zebrafish favorece a expressão de transgenes fluorescentes e torna-se fácil monitorar a expressão e atividade de muitos genes (GULATI-LEEKHA & GOLDMAN, 2006), tornando-os suscetíveis à manipulação e à microinjeção (LELE & KRONE, 1996).

Somados a estes aspectos, outros atributos práticos fazem com que o zebrafish seja atraente para estudos de laboratório quando comparado a outros modelos, tais como a mosca-das-frutas (*Drosophila melanogaster*), o rato (*Rattus norvegicus*) e o camundongo (*Mus musculus*). Algumas vantagens que podem ser destacadas são o pequeno espaço requerido para a manutenção, o baixo custo e a capacidade de armazenamento de amostras biológicas (LIESCHKE & CURRIE, 2007). Portanto, o zebrafish é um animal que combina a relevância de um vertebrado na escala de um invertebrado (GOLDSMITH, 2004).

Nos últimos anos está acontecendo um progresso considerável na genética e genômica do zebrafish (POSTLETHWAIT et al., 2000). Em 2001, o Instituto Sanger começou o seqüenciamento do genoma desta espécie (VOGEL, 2000; STERN & ZON, 2003). Paralelamente ao projeto genoma do zebrafish está ocorrendo também o seqüenciamento e identificação dos ESTs. A seqüência do genoma mitocondrial já é conhecida, servindo de base para estudos filogenéticos (BROUGHTON et al., 2001). O estudo do genoma do zebrafish pode servir como um complemento funcional para o projeto genoma humano, o qual produz enormes quantidades de seqüências, porém carece de informações funcionais para a maioria dos genes identificados (DOOLEY & ZON, 2000). Além disso, os genes deste teleósteo são evolutivamente conservados e apresentam um alto grau de similaridade com os genes humanos e de camundongo (BARBAZUK et al., 2000; LISCHKE & CURRIE, 2007). Entretanto, em algum momento da evolução dos teleósteos, houve um evento completo de duplicação genômica, fato que não ocorreu entre os mamíferos (BRUNET et al., 2006). Mesmo que apenas uma pequena porção destas duplicações gênicas ainda permaneça, é bastante comum a presença de mais de um gene de função homóloga, chamados de parálogos (AMORES et al., 1998; TAYLOR et al., 2003).

O interesse pelo zebrafish pode ser observado pelo vasto número de laboratórios que utilizam este teleósteo como modelo experimental em suas pesquisas (SPRAGUE et al., 2001) e pelo crescimento exponencial do número de estudos publicados que envolvem esta espécie (ZON & PETERSON, 2005; LIESCHKE & CURRIE, 2007). Foi criada uma rede de informações na web sobre o zebrafish, o ZFIN (http://zfin.org), na qual laboratórios do mundo inteiro podem depositar um grande número de informações sobre esta espécie (SPRAGUE et al., 2003). Além disso, existe um excelente, compreensivo e freqüentemente atualizado manual de manutenção e controle das condições ideais para a criação deste teleósteo em laboratórios (WESTERFIELD, 2000).

Numerosos avanços em triagens genéticas têm sido obtidos com o zebrafish. Milhares de mutações distintas estão sendo identificadas, e mais de 400 delas têm sido clonadas (AMSTERDAM et al., 2004; SCHONTHALER et al., 2008). Embora estas triagens genéticas sejam mais informativas a respeito da embriogênese do zebrafish, a identificação de genes correlacionados com patologias humanas tem sido efetivada nesta espécie (ZON & PETERSON, 2005; LIESCHKE & CURRIE, 2007). Dentre eles, podem ser citados genes envolvidos na síndrome do rim policístico (OTTO et al., 2003), metabolismo do colesterol (FARBER et al., 2001), regeneração tecidual (POSS et al., 2003), malformações cardíacas (GARRITY et al., 2002), anemias (DONOVAN et al., 2000), câncer (AMATRUDA et al., 2002; LANGENAU et al., 2003) e transtornos no SNC (LI & DOWLING, 1997). Além disso, já foram identificados muitos tipos de neoplasias no zebrafish, as quais são semelhantes histologicamente e geneticamente com às de humanos, o que mostra que a biologia do câncer é similar nestes organismos (AMATRUDA et al., 2002; STERN & ZON, 2003). A geração de animais transgênicos pode ser efetivada com alterações em genes específicos relacionados ao câncer (LONG et al., 1997).

Atualmente, a utilização do zebrafish vem sendo expandida para outras áreas do conhecimento, tais como bioquímica (TAYLOR et al., 2004), neurociência (EDWARDS & MICHEL, 2002), toxicologia (HILL et al., 2005), farmacologia (GOLDSMITH et al., 2004) e biologia do comportamento (GERLAI, 2003; GUO, 2004). Devido às suas peculiaridades reprodutivas e à presença de semelhanças morfológicas e fisiológicas com mamíferos, esta espécie desperta o interesse pela oportunidade de acelerar o processo da descoberta de novas drogas (STERN & ZON, 2003). Este teleósteo é capaz de absorver de forma rápida os compostos que são diretamente adicionados na água e acumulá-los em diferentes tecidos, principalmente no SNC (GROSSEL & WOOD, 2002). Por apresentar um tamanho relativamente pequeno, a quantidade de compostos a ser testada passa a ser relativamente menor, contribuindo com a otimização do uso das drogas de estudo (YAMAZAKI et al., 2002; GOLDSMITH, 2004). Dentre os trabalhos envolvendo aspectos toxicológicos, a exposição a diferentes contaminantes ambientais, tais como a 2,3,7,8-tetraclorodibenzeno-*p*-dioxina (TCDD) (DONG et al., 2002; HILL et al., 2003), pesticidas carbamatos e organofosforados (SENGER et al., 2005), metanol (RICO et al., 2006), etanol (RICO et al., 2008) e metais pesados (SENGER et al., 2006; ROSEMBERG et al., 2007a) já foi estudada no SNC de zebrafish.

Exposições a drogas de abuso, tais como etanol e nicotina já estão sendo estudadas neste teleósteo (GERLAI et al., 2006; RICO et al., 2007; LEVIN et al., 2007; RICO et al., 2008). Evidências têm demonstrado que o tratamento com etanol é capaz de modificar diversos parâmetros comportamentais no zebrafish (GERLAI, 2003, GERLAI et al, 2006). Dentre estes, podem ser destacados a atividade locomotora, a interação social, a agressividade e o comportamento antipredatório (GERLAI et al., 2000). Estudos envolvendo exposição crônica ao etanol têm sido realizados e os resultados corroboram a hipótese do zebrafish ser um excelente modelo vertebrado que mimetiza aspectos característicos do alcoolismo (GERLAI et al, 2006). Além disto, existe um amplo espectro de paradigmas comportamentais complexos já descritos para este vertebrado (NINKOVIC & BALLY-CUIF, 2006).

O zebrafish também propicia a realização de muitos estudos para a compreensão das bases moleculares da neurobiologia, identificando genes envolvidos na formação de circuitos neuronais, no comportamento e nos mecanismos envolvidos na neuropatogênese (VASCOTTO et al., 1997; GUO, 2004). Muitos sistemas de neurotransmissão já foram identificados nesta espécie tais como: glutamatérgico (EDWARDS & MICHEL, 2002); colinérgico (BEHRA et al., 2002; CLEMENTE et al., 2004; ARENZANA et al., 2005); dopaminérgico (RYU et al., 2006); serotoninérgico (LILLESAAR et al, 2007); histaminérgico (KASLIN & PANULA, 2001); gabaérgico (KIM et al., 2004); purinérgico (KUCENAS et al., 2003; RICO et al., 2003; SENGER et al., 2004).

I.4.2. Sistema purinérgico

O sistema purinérgico é caracterizado por ser uma via de sinalização importante para diferentes tecidos, sendo capaz de desencadear múltiplos efeitos celulares (BURNSTOCK, 2006). Além de sua função energética, o ATP atua como uma molécula sinalizadora e, juntamente com outros nucleotídeos extracelulares, modula múltiplas funções tais como desenvolvimento, fluxo sangüíneo, secreção, inflamação e respostas imunológicas (ROBSON et al., 2006). Estudos recentes têm demonstrado que este nucleotídeo pode desempenhar um papel na vasodilatação em células endoteliais (HARRINGTON et al., 2007) e estar envolvido na oscilação dos níveis de Ca^{2+} em culturas de miócitos (NAKAYAMA et al., 2007), além de atuar como um neurotransmissor no SNC (BURNSTOCK, 2007; ZIMMERMANN, 2007).

A descrição inicial do ATP como molécula neurotransmissora ocorreu através dos estudos que mostravam a sua liberação a partir de nervos sensoriais (HOLTON & HOLTON, 1954 e HOLTON, 1959). Entretanto, sua ação como neurotransmissor só foi reconhecida pelos estudos realizados pelo grupo de Geoffrey Burnstock e colaboradores, que desenvolveram a hipótese purinérgica (BURNSTOCK et al., 1970; BURNSTOCK, 1972). Estudos demonstram que o ATP é co-liberado na fenda sináptica juntamente com outros neurotransmissores, tais como: acetilcolina, glutamato, norarenalina, serotonina e GABA (BURNSTOCK, 2004; BURNOSTOCK, 2007; ZIMMERMANN, 2007).

Os nucleotídeos e o nucleosídeo da adenina podem exercer seus efeitos através da ativação de receptores purinérgicos subdivididos em dois grandes grupos: P1 e P2. Os purinoreceptores do tipo P1 são mais eficientemente ativados por adenosina, enquanto que os purinoreceptores P2 são ativados por ATP (RALEVIC & BURNSTOCK, 1998). Estes receptores são denominados purinoceptores P2 e são divididos em duas subclasses, os receptores ionotrópicos P2X, que são canais iônicos dependentes de ligantes, e os receptores metabotrópicos P2Y, que são acoplados à proteína G. Membros de ambos os tipos de receptores encontram-se amplamente distribuídos e estão envolvidos em uma miríade de funções (BARNARD et al., 1997; BURNSTOCK & KNIGTH, 2004).

O controle da sinalização mediada por ATP é exercido por enzimas de membrana denominadas ecto-nucleotidases (BONAN et al., 2001). Este conjunto de enzimas inclui a família das E-NPP (ectonucleotídeo pirofosfatase/fosfodiesterase), a família das NTPDases (nucleosídeo trifosfato difosfoidrolases), as fosfatases alcalinas e a ecto-5’-

nucleotidase (EC 3.1.3.5) (ROBSON et al., 2006). A cascata de hidrólise do ATP culmina na formação do nucleosídeo adenosina.

Em zebrafish, já foram identificados diferentes tipos de receptores P2 (DIAZ-HERNANDEZ et al., 2002; KUCENAS et al., 2003). Além disso, a hidrólise de nuclotídeos já foi caracterizada no SNC desta espécie (RICO et al., 2003; SENGER et al., 2004), bem como a presença de diferentes membros da família das NTPDases (RICO et al., 2006; APPELBAUM et al., 2007) e de uma ecto-5'-nucleotidase (ROSEMBERG et al., 2007a), a qual hidrolisa o AMP até adenosina.

I.4.3. Adenosina

A adenosina (ADO) é um nucleosídeo que pode ser sintetizado tanto no meio intracelular quanto no espaço extracelular. A síntese intracelular é devida principalmente à ação da enzima 5'-nucleotidase que hidrolisa AMP à adenosina e da hidrólise do substrato S-adenosil-homocisteína pela S-adenosil-homocisteína hidrolase. A adenosina produzida intracelularmente pode ser transportada ao espaço extracelular através de transportadores bidirecionais, por um mecanismo de difusão facilitada que regula os níveis intracelulares e extracelulares deste nucleosídeo (FREDHOLM et al., 2005). Suas ações são exercidas através de um grupo de receptores metabotrópicos, que estão divididos em quatro subtipos: A_1, A_{2A}, A_{2B} e A_3. Grande parte do conhecimento sobre a distribuição e funcionalidade dos purinoreceptores P1 corresponde a estudos referentes aos receptores A_1 e A_{2A} (FREDHOLM et al., 2001; BURNSTOCK, 2006).

A interação da ADO com seus respectivos purinoreceptores desempenha diversas funções na sinalização celular, podendo atuar na neuromodulação, neuroproteção, secreção endócrina e exócrina, agregação plaquetária, vasodilatação e nocicepção

(BURNSTOCK, 2006). A sinalização adenosinérgica pode ser controlada pelo transporte bidirecional seguido por fosforilação até AMP pela adenosina cinase (AK) ou pelo mecanismo de desaminação à inosina (INO), promovido pela adenosina deaminase (ADA) tanto no meio extracelular quanto intracelular (FREDHOLM et al., 2005).

I.4.4. Adenosina deaminase

A adenosina deaminase (ADA, EC 3.5.4.4) é responsável por promover a desaminação hidrolítica da ADO em INO. Além deste nucleosídeo, a 2’-desoxiadenosina (2’-D-ADO) é outro substrato capaz de ser clivado pela ADA, sendo convertido a 2’-desoxinosina (2’-D-INO) (IWAKI-EGAWA et al., 2004).

Esta enzima possui uma função importante no sistema imune em processos inflamatórios, controlando os níveis de ADO (ZAVIALOV & ENGSTRÖM, 2005; ANTONIOLI et al., 2007). Estudos têm sugerido que a INO não é apenas um metabólito inativo, mas que pode ter ação anti-inflamatória (HASKÓ et al., 2000) e neuroprotetora em injúrias cerebrais (TSUDA, 2005).

A disfunção do metabolismo da ADO causada pela deficiência da ADA nos sistemas biológicos pode acarretar a síndrome da imunodeficiência severa combinada (SIDSC), ocasionando lesões e problemas funcionais de muitos órgãos com a alteração do metabolismo de purinas (MORTELLARO et al., 2006). Estudos realizados com camundongos nocautes para o gene da ADA têm demonstrado que a terapia gênica *ex vivo* utilizando vetores lentivirais são eficazes na prevenção e correção dos fenótipos da SIDSC, o que dá suporte a futuros estudos clínicos envolvendo esta patologia (ARIGA, 2006; CARBONARO et al., 2006; MORTELLARO et al., 2006). Além disso, a determinação da atividade cinética da ADA demonstra uma significativa correlação com a proliferação de células do sistema imune e a densidade de linfócitos-T *in vitro* (KAINTHLA et al., 2006).

Dois membros clássicos da ADA estão descritos, sendo eles primeiramente denominados como ADA1 e ADA2 (HIRSCHHORN & RATECH., 1980). Estudos recentes têm demonstrado que ambos os membros apresentam características cinéticas distintas, o que faz com que ambos possam desempenhar uma função diferenciada nos organismos (IWAKI-EGAWA et al., 2004; ZAVIALOV & ENGSTRÖM, 2005).

A ADA1 é uma enzima monomérica, cuja massa molecular é de aproximadamente 30-40 kDa (DADDONA & KELLEY, 1977). A deficiência inata desta enzima no sistema imune está diretamente relacionada com a SIDSC e a ausência de linfócitos T e B funcionais em indivíduos afetados (MORTELLARO et al., 2006). Tecidos como fígado e rins apresentam tanto a ADA1 solúvel quanto a forma associada a uma proteína de ligação, sendo que esta é constituída por duas moléculas de ADA1 e uma proteína ligante, com um K_A aproximado de 4-20 nM (SCHRADER et al., 1990; IWAKI-EGAWA & WATANABE, 2002). O complexo ADA-proteína de ligação constitui uma ecto-ADA, a qual é responsável pelo controle dos níveis de ADO extracelular (SAURA et al., 1996; TORVINEN et al., 2002). Evidências demonstram que esta proteína de ligação é uma glicoproteína multifuncional do tipo II, conhecida como CD26 ou dipeptidil peptidase IV (DPPIV), a qual pode ser encontrada na forma solúvel ou integrada à membrana (GORRELL et al., 2001; GORRELL, 2005). Além de ser uma proteína de ligação da ADA1, a CD26 possui atividade peptidásica e funciona como uma molécula de adesão à matriz extracelular, podendo influenciar processos de sinalização celular (GORRELL et al., 2001). Entretanto, a atividade extracelular de desaminação de ADO não depende da ligação da ADA1 com CD26, uma vez que está demonstrado que em ratos e camundongos esta proteína não funciona como um ligante da ADA1 em membranas (IWAKI-EGAWA et al., 1997). O sítio de ligação com CD26 depende de alguns resíduos de aminoácidos carregados presentes na ADA1, como Arg-142, Glu-139 e Asp-143, os quais estão presentes em humanos, mas não nesses roedores (RICHARD et al., 2002).

Estudos envolvendo a sinalização mediada pela ADO no sistema nervoso central (SNC) demonstram que além da interação com CD26, a ADA1 pode funcionar como uma ecto-enzima ancorada aos receptores de adenosina A_1 e A_2B, mediando os processos de sinalização deste neuromodulador (CIRUELA et al., 1996; FRANCO et al., 1997; ROMANOWSKA et al., 2007). Desta maneira, a ecto-ADA poderia exercer um importante papel na comunicação entre células neuronais e gliais, regulando o nível do ligante ADO para os receptores P_1 (FRANCO et al., 1997). Na literatura, existem evidências de que ADA1, receptores A_1 e CD26 estão co-localizados em cérebro de goldfish (*Carassius auratus*), sugerindo que a ecto-ADA pode apresentar uma estrutura bastante complexa em peixes (BERAUDI et al., 2003).

Diferentemente da ADA1, a ADA2 apresenta diferenças, tanto estruturais quanto cinéticas. Sua massa molecular é de aproximadamente 100 kDa e representa uma menor parte da atividade da ADA em tecidos, sendo abundante em plasma (IWAKI-EGAWA et al, 2004; RATECH & HIRSCHHORN, 1981). A atividade desta enzima encontra-se elevada em casos de doenças hepáticas; entretanto, a fonte celular e a função da ADA2 plasmática ainda não estão completamente esclarecidas (KOBAYASHI et al., 1993). Dados recentes têm sugerido que ela pode ser secretada por monócitos ativados em processos inflamatórios (IWAKI-EGAWA et al., 2006). A purificação, caracterização e determinação da seqüência N-terminal da ADA2 do fígado de galinha demonstrou que esta enzima possui um pH ótimo na faixa do 6,5 e que também é *N*-glicosilada, tendo uma seqüência distinta à da ADA1 (IWAKI-EGAWA et al., 2004). Estudos com a ADA2 em humanos têm demonstrado que ela está agrupada em uma família de fatores de crescimento relacionados à adenosina deaminase (ADGF) e é codificada por um gene localizado no cromossomo 22 de humanos, conhecido por *CECR1* "cat-eye syndrom critical region 1" (ZAVIALOV & ENGSTRÖM, 2005). A duplicação deste cromossomo, principalmente do gene *CECR1*, origina a síndrome do olho de gato, a qual é um raro defeito no desenvolvimento capaz de acarretar modificações severas no coração, rim e olhos (RIAZI et al., 2005). A família dos genes que codificam diversos ADGFs já foi estudada em *Drosophila*, sendo evidenciadas diferenças na expressão destes genes durante as fases do desenvolvimento deste invertebrado (MAIER et al., 2001). A atividade da ADGF nas glândulas salivares do inseto hematófago *Lutzomia longipalpis* já foi caracterizada, demonstrando que este fator de crescimento pode exercer suas ações através da depleção de ADO (CHARLAB et al., 2001). Estudos de comparação de seqüência demonstraram que em zebrafish existem dois genes parálogos da ADA2, chamados de *CECR1-1* e *CECR1-2*, os quais estão agrupados consistentemente com a ADGF de outros organismos (MAIER et al., 2001; MAIER et al., 2005).

Embora a existência de dois membros da ADA fosse previamente consolidada, uma análise filogenética das seqüências de diferentes organismos revelou uma nova família de proteína relacionada com a ADA1 e ADA2, a qual foi denominada ADAL. Todos estes membros, juntamente com a adenina deaminase (ADE) presente em alguns fungos e bactérias e a adenosina-5'-monofosfato deaminase (AMPD) foram classificados como subfamílias pertencentes ao grupo das adenil-deaminases (MAIER et al., 2005). Por apresentar sítios de aminoácidos importantes relacionados à desaminação de ADO e motivos conservados entre as subfamílias da ADA, é sugerido que a ADAL também pode participar da clivagem de ADO à INO (MAIER et al., 2005). Contudo, não existem estudos demonstrando a atividade ou expressão deste membro das adenil-deaminases em nenhum modelo animal até o presente momento.

I.4.5. Objetivos

Considerando que: (1) o zebrafish é um importante e consolidado modelo experimental em estudos envolvendo genética e neuroquímica; (2) a ADO é um produto que pode ser produzido através da hidrólise dos nucleotídeos da adenina, a qual já foi previamente caracterizada nesta espécie; (3) a ADA representa um dos mecanismos de inativação da sinalização adenosinérgica, controlando os níveis de ADO e INO, o objetivo geral deste estudo foi realizar um mapeamento de expressão tecidual dos genes relacionados à ADA e caracterizar a atividade de desaminação da ADO em cérebro de zebrafish.

Objetivos específicos:

- Identificar os membros pertencentes às diferentes subfamílias relacionadas à ADA em zebrafish.
- Analisar as semelhanças e diferenças entre as seqüências deduzidas de aminoácidos dos diferentes membros da ADA de zebrafish, bem como verificar possíveis resíduos importantes para o funcionamento destas enzimas.
- Verificar o padrão transcricional dos genes relacionados à ADA em diferentes tecidos de zebrafish, tais como cérebro, brânquias, coração, fígado músculo esquelético e rins.

Em frações solúvel e de membrana de cérebro de zebrafish:

- Padronizar as condições ótimas de tempo e quantidade de proteína para os ensaios das atividades de desaminação da ADO.
- Determinar as propriedades bioquímicas da ADA, tais como pH ótimo, influência de cátions divalentes, constantes cinéticas (K_M e V_{max}) e desaminação de nucleosídeos derivados da adenina e da guanina.
- Verificar o efeito do EHNA sobre a atividade de desaminação de ADO.

PARTE II

CAPÍTULO 1

Denis Broock Rosemberg, Eduardo Pacheco Rico, Marcus Rodrigo Guidoti, Renato Dutra Dias, Diogo Onofre Souza, Carla Denise Bonan, Maurício Reis Bogo.

Adenosine deaminase-related genes: molecular identification, tissue expression pattern and truncated alternative splice isoform in zebrafish (*Danio rerio*).

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Adenosine deaminase-related genes: Molecular identification, tissue expression pattern and truncated alternative splice isoform in adult zebrafish (*Danio rerio*)

Denis Broock Rosemberg [a], Eduardo Pacheco Rico [a], Marcus Rodrigo Guidoti [b], Renato Dutra Dias [b], Diogo Onofre Souza [a], Carla Denise Bonan [b,*], Maurício Reis Bogo [c,*]

[a] *Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil*
[b] *Laboratório de Neuroquímica e Psicofarmacologia, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil*
[c] *Laboratório de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil*

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Abstract

Adenosine deaminase (ADA) is responsible for cleaving the neuromodulator adenosine to inosine. Two members of ADA subfamilies, known as ADA1 and ADA2, were described and evidence demonstrated another similar protein group named ADAL (adenosine deaminase "like"). Although the identification of ADA members seems to be consistent, the expression profile of *ADA1*, *ADA2* and *ADAL* genes in zebrafish has not yet been reported. The aim of the present study was to map the expression pattern of ADA-related genes in various tissues of adult zebrafish (*Danio rerio*). An extensive search on zebrafish genome followed by a phylogenetic analysis confirmed the presence of distinct ADA-related genes (*ADA1*, *ADAL* and two orthologous genes of *ADA2*). Specific primers for each ADA member were designed, optimized semi-quantitative RT-PCR experiments were conducted and the relative amount of transcripts was determined. The tissue samples (brain, gills, heart, liver, skeletal muscle and kidney) were collected and the expression of *ADA1*, *ADAL* and *ADA2* genes was characterized. *ADA1* had a similar expression pattern, whereas *ADAL* was less expressed in the heart. The highest relative amount of *ADA2-1* transcripts was observed in the brain, liver and gills and it was less expressed in the heart. RT-PCR assays revealed that the other ADA2 form (*ADA2-2*) was expressed ubiquitously and at comparable levels in zebrafish tissues. The strategy adopted also allowed the identification of an *ADA2-1* truncated alternative splice isoform (*ADA2-1/T*), which was expressed at different intensities. These findings demonstrated the existence of different ADA-related genes, their distinct expression pattern and a truncated *ADA2-1* isoform, which suggest a high degree of complexity in zebrafish adenosinergic system.

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Keywords: Adenosine deaminase; ADA-related genes; Zebrafish; Adenosinergic system

Introduction

Adenosine is a nucleoside that exerts several actions in many tissues. Its effects are elicited by specific P1 metabotropic receptors, named A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al., 2005). The inhibitory actions of adenosine are mediated by A_1 and A_3, whereas facilitatory mechanisms involve the activation of A_{2A} and A_{2B} receptors (Latini and Pedata, 2001; Rebola et al., 2005). This nucleoside plays several roles in the fast purinergic signaling among tissues, which include neuromodulation, exocrine and endocrine secretion, platelet aggregation, vascular endothelial cell-mediated vasodilatation and nociceptive mechanosensory transduction (Burnstock, 2006). Extracellular

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* Corresponding authors. Tel.: +55 51 3320 3500x4726x4158; fax: +55 51 3320 3568.
E-mail addresses: cbonan@pucrs.br (C.D. Bonan), mbogo@pucrs.br (M.R. Bogo).

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adenosine concentrations may increase dramatically after seizures, hypoxia and ischemia, playing neuroprotective roles (Cunha, 2001; Dunwiddie and Masino, 2001). Extracellular concentrations of adenosine might be regulated by neural cell uptake through bidirectional nucleoside transporters (Pinto-Duarte et al., 2005) and ecto-nucleotidase activity (Latini and Pedata, 2001). Adenosine uptake followed by its phosphorylation to AMP by adenosine kinase (AK) or deamination to inosine by adenosine deaminase (ADA) are two possible mechanisms able to promote the inactivation of adenosine signaling (Latini and Pedata, 2001; Fredholm et al., 2005).

Adenosine deaminase (ADA, EC 3.5.4.4) is an important enzyme that promotes the irreversible hydrolytic deamination of adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine, respectively. Although inosine had been considered an inactive metabolite, recent evidence suggested that it may exert a function as a natural antioxidant and radioprotector for mice exposed to lethal doses of gamma-radiation (Gudkov et al., 2006) with antiinflammatory (Haskó et al., 2000) and neuroprotective actions against brain injury (Tsuda, 2005).

There are different ADA members in animal cells. These enzymes belong to the adenyl-deaminase family, being grouped consistently with AMP deaminase (AMPD) and adenine deaminase (ADE) as distinct subfamilies (Maier et al., 2005). In humans, it has been shown that almost all ADA activity is attributed to ADA1 (Zavialov and Engstrom, 2005). Deficiency of this protein may lead to severe combined immunodeficiency (SCID) in childhood due to the importance of ADA1 in the immune response mediated by T-lymphocytes (Pacheco et al., 2005; Ozdemir, 2006). This enzyme may be found anchored to the cell membrane in a complex with CD26 (DPPIV — dipeptidyl-peptidase IV) as an ecto-ADA, responsible to breakdown extracellular adenosine (Franco et al., 1997). Studies have demonstrated that this ADA form is also colocalized with A_1 receptors in CNS, suggesting that the modulation of adenosine signaling through A_1R is mediated in part by ecto-ADA in neuronal and non-neuronal cells (Franco et al., 1998; Ruiz et al., 2000).

The other member, ADA2, is most abundant in human plasma and has different kinetic properties when compared to ADA1 (Iwaki-Egawa et al., 2006). This ADA member had already been purified and characterized in chicken liver (Iwaki-Egawa et al., 2004). Evidence has shown that ADA2 belongs to a new family of growth factors named adenosine deaminase-related growth factors (ADGFs) with ADA activity, encoded by cat-eye syndrome critical region candidate 1 (*CECR1*) gene in vertebrates (Zavialov and Engstrom, 2005).

Study performing a phylogenetic analysis of ADA subfamilies revealed that there is another protein with high similarity to the classic members of ADA, however, it is consistently grouped in a different manner. This member was named ADAL (adenosine deaminase "like") and its genetics, biochemical and physiological properties are still unknown. It has been speculated that it may present ADA activity, because ADAL deduced amino acid sequence has catalytically important sites (Maier et al., 2005).

Zebrafish (*Danio rerio*) is a teleost widely used as a vertebrate model in biochemical, genetic and neurochemical studies (Grunwald and Eisen, 2002; Lieschke and Currie, 2007) and the genome of this fish shares many similarities with the human genome (Lieschke and Currie, 2007). Purinergic signaling studies have already been performed in zebrafish (Kucenas et al., 2003) and ecto-nucleotidases, enzymes able to form extracellular adenosine through ATP catabolism, were recently characterized in zebrafish brain membranes (Rico et al., 2003; Senger et al., 2004). Moreover, it has been demonstrated that these enzymes may be differently modulated by contaminants, such as pesticides and metals (Senger et al., 2005; Rosemberg et al., 2007) and it has been shown that NTPDase1 and three distinct forms of NTPDase2 are able to contribute for ATP and ADP hydrolysis in zebrafish brain (Rico et al., 2006).

The expression pattern of murine *ADA1* had already been determined in different tissues (Chinsky et al., 1989) and the amount of *ADA2* transcripts had been also investigated during *Drosophila* development stages (Maier et al., 2001) and in the cabbage armyworm, *Mamestra brassicae* (Zhang and Takeda, 2007). However, as ADA1, ADA2 and ADAL are grouped in three ADA subfamilies, it is possible that they might have a distinct expression pattern, which could reveal a different contribution for deamination activity among tissues. Thus, the aim of the present study was to map the expression profile of *ADA1*, *ADA2* and *ADAL* genes in different tissues from adult zebrafish to show how the ADA genes could be a fine-tuning regulation for adenosine activity in the zebrafish adenosinergic system.

Materials and methods

Animals

Adult wild-type zebrafish (*D. rerio*) of both sexes (around 3 months old) were obtained from commercial supplier and acclimated for 2 weeks in a 50-L thermostated aquarium. The water was kept between 26±2 °C under a 12-h light–dark controlled photoperiod and the animals were fed with commercial fish pellet twice a day. The use and maintenance of zebrafish were according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

Sequences analysis and primers design

The identification of ADA1, ADA2 and ADAL was performed in NCBI Blast searches of GenBank, using the *Homo sapiens*, *Mus musculus*, *Gallus gallus* and *Xenopus laevis* proteins as queries. The obtained sequences (supported by mRNA or EST data) were compared with zebrafish protein database at Zebrafish Information Network (ZFIN) (University of Oregon, Eugene, OR 97403-5274; World Wide Web URL: http://zfin.org) and the alignment was performed using ClustalX program (Thompson et al., 1997). A phylogenetic tree was constructed according to Neighbor-Joining method (Saitou and Nei, 1987) using proportional (*p*) distance with MEGA 2.1 program (Ryu et al., 2002).

In order to compare the zebrafish deduced amino acid sequences, an alignment was performed using ClustalX. To minimize problems due to the sequence divergence observed between ADA1/ADAL and ADA2, manual adjustments were made using the BioEdit 7.0.9 program. The sequences were analyzed using the tools available at CBS Prediction Servers (http://www.cbs.dtu.dk/services/).

Zebrafish DNA sequences encoding to each one of ADA members were retrieved from GenBank database and aligned using ClustalX program. Regions with low scores of similarity among the sequences were used for searching specific primers, which were designed using the program Oligos 9.6. In order to confirm the primers specificity, each primer was compared with zebrafish genome and it was able to recognize only its specific target sequence. Thus, the strategy adopted to construct the primers did not allow cross-amplification. The *β-actin* primers were designed as described previously (Chen et al., 2004) and the optimal PCR conditions were determined (Table 1).

Reverse transcription-polymerase chain reaction (RT-PCR)

Zebrafish brain, gills, heart, liver, skeletal muscle and kidney were dissected under sterile conditions and immediately frozen in liquid nitrogen. RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. In order to achieve a similar weight (around 50 mg) from different tissues, the number of animals used for the experiments was: brain ($n=5$), gills ($n=5$), heart ($n=10$), liver ($n=5$), muscle ($n=5$) and kidney ($n=12$). In brief, the tissue samples were homogenized in 500 µl of TRIzol. Chloroform (100 µl) was added and the mixture was centrifuged at 10.600 ×*g* for 10 min at 5 °C. The upper aqueous phase was collected and the RNA was precipitated by addition of isopropyl alcohol (250 µl), followed by a centrifugation at 10.600 ×*g* for 10 min at 5 °C. The pellets were further washed with 500 µl of 75% cold ethanol and centrifuged at 6.800 ×*g* for 5 min at 5 °C. The samples were dried out and resuspended in 15 µl RNase-free water. Before storing at −70 °C, 0.4 µl of RNaseOUT Ribonuclease Inhibitor (Recombinant) (Invitrogen, USA) was added. RNA purity was quantified spectrophotometrically calculating the ratio between absorbance values at 260 and 280 nm and 2 µl were tested by electrophoresis in a 1.0% agarose gel containing ethydium bromide. All samples were adjusted to 160 ng/µl and cDNA species were synthesized with SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen, USA). Each RNA sample was mixed with 1 µl of 50 µM Oligo (dt) and 1 µl Annealing Buffer (final volume of 8 µl), being incubated in a thermal cycler at 65 °C for 5 min. Immediately, the samples were placed on ice for 1 min and 10 µl 2X First-Strand Reaction Mix and 2 µl SuperScript™ III/RNaseOUT™ Enzyme Mix were added. The products were mixed, incubated by 50 min at 50 °C and the reaction was finished at 85 °C for 5 min.

RT-PCR conditions were optimized in order to determine the number of cycles that would allow product detection within the linear phase of mRNA transcripts amplification. The reaction for *β-actin* gene was performed using in a total volume of 20 µl, 0.1 µM primers (Table 1), 0.2 µM dNTP, 2 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (Invitrogen, USA). *ADA1* and *ADAL* PCR assays were performed in a total volume of 25 µl, 0.08 µM primer (Table 1), 0.2 µM dNTP, 3 mM $MgCl_2$ and 1 U Taq DNA polymerase. *ADA2-1* PCR reactions were performed at these same conditions, except that 2 mM $MgCl_2$ was employed. The amplification of *ADA2-2* was carried out at the same condition of *ADA1* and *ADAL*. The *ADA2-2* PCR assay was optimized using 0.5 M betain and 1 U Taq Platinum DNA polymerase (Invitrogen, USA). *ADA1* and *ADA2-2* PCR assays were carried out using 2 µl cDNA as template, whereas *ADA2-1*, *ADAL* and *β-actin* were conducted using 1 µl cDNA. The following conditions were used for PCR reactions: 1 min at 94 °C, 1 min for annealing temperature (see Table 1), 1 min at 72 °C for 28 cycles (*ADA1*) and 35 cycles (*ADAL*, *ADA2-1*, *ADA2-2* and *β-actin*). A postextension cycle at 72 °C was performed for 10 min. For each set of PCR reactions, a negative control was included. PCR products were resolved by a 1.0% agarose gel containing ethydium bromide and visualized with ultraviolet light. The fragments length of PCR reactions was confirmed with Low DNA Mass Ladder (Invitrogen, USA) and *β-actin* was carried out as an internal standard. The relative abundance of each mRNA versus *β-actin* was determined in the organs studied by densitometry using the freeware ImageJ 1.37 for Windows.

Each experiment was repeated four times using RNA isolated from independent extractions. The expression analysis in each tissue was performed in replicate and representative findings were shown. The normalized expression levels of ADA-related genes at different tissues were expressed as mean ± S.E. and statistically compared by one-way analysis of variance

Table 1
Primer sequences and PCR amplification conditions

ADA member	GenBank accession number	ZFIN ID	Primers (5′–3′)	T_m (°C)	Cycles
ADA1	AAH76532	ZDB-GENE-040718-393	F — CAGGTCCATTCTGTGCTGCATGCGTC R — AAGTGTGTGGTATCCGTGCCCAATGC	58	28
ADA2-1 ([a])	AAL40922	ZDB-GENE-030902-4	F — AAGACAAGGGTTTTAACCTGCCCTAC R — CTCCTTTCTTTGACTTGGCAATGTGC	63	35
ADA2-2	XP_687719	ZDB-GENE-041210-77	F — CTGAAGATGAAGGAAATCACCCTTTCACC R — TGTCTTCATAAAGCTCTTTCAAACCCTGG	54	35
ADAL	NP_001028916	ZDB-GENE-050913-145	F — TCATTCAAGAGTTTGCGGCAGATGG R — TTGGCTTTCTGAAGTGCAGCGAGC	61	35
β-actin	AAC13314	ZDB-GENE-000329-1	F — GTCCCTGTACGCCTCTGGTCG R — GCCGGACTCATCGTACTCCTG	54	35

[a] The same primers amplified a truncated *ADA2-1* splice isoform (*ADA2-1/T*).

(ANOVA), followed by Tukey HSD test as post-hoc. *P* values ≤ 0.05 were considered significant.

Results

Identification of zebrafish ADA orthologous genes

Proteins of *H. sapiens*, *M. musculus*, *G. gallus* and *X. laevis* were retrieved from GenBank and used as queries for identification of zebrafish ADA-related orthologous genes. These organisms presented similar sequences to each one of ADA members except *M. musculus*, which had no similar protein to ADA2. The searches resulted in ADA1 (AAH76532), ADA2-1 (AAL40922), ADA2-2 (XP_687719) and ADAL (NP_001028916) similar deduced amino acid sequences in zebrafish. In order to confirm the sequences identity and to achieve the information about the current data annotation, these sequences were compared with zebrafish protein database at ZFIN (Table 1).

The phylogenetic tree was constructed using Neighbor-Joining method and proportional (*p*) distance (Fig. 1). Three well-resolved terminal clades supported by high bootstrap values were identified. The first clade grouped the AAH76532 zebrafish sequence with all other ADA1 sequences, whereas NP_001028916 was included in ADAL clade. The AAL40922 and XP_687719 sequences were consistently grouped in the ADA2 clade. The tree topology strongly suggests homologous functions on zebrafish genome.

The protein characteristics of zebrafish ADA1, ADAL, ADA2-1, and ADA2-2 were investigated (Fig. 2). The deduced amino acid sequences alignment showed the eight conserved domains among the three ADA proteins subfamilies and the ADA2-1 and ADA2-2 signal peptides, as described previously (Maier et al., 2005). Furthermore, it was possible to verify that ADA1 and ADAL sequences presented only one potential *N*-glycosylation site, whereas ADA2-1 and ADA2-2 presented five and four sites, respectively. High score putative phosphorylation residues were also investigated using NetPhosk, a kinase-specific prediction of protein phosphorylation sites tool (Blom et al., 2004). The results identified two potential phosphorylation sites for ADA1: Ser40 residue for Protein Kinase A (PKA) and the Thr285 for Protein Kinase C (PKC). The ADAL sequence analysis revealed a putative phosphorylation site at Thr153 residue for PKC. ADA2-1 had the Thr132, Thr284, Ser479 and Ser480 residues as potential PKC targets, whereas ADA2-2 sequence presented Ser60 and Ser165 residues for PKA and Ser291 for PKC. Considering the conserved cysteine residues, it was possible to identify four residues for ADA2-1 and ADA2-2 and only one conserved cysteine residue for ADA1 and ADAL amino acid sequences. Neither hydrophobic transmembrane domains nor potential N-terminal acetylation sites (Kiemer et al., 2005) were identified for zebrafish ADA1, ADAL, ADA2-1 and ADA2-2 proteins.

Gene expression pattern of ADA members in zebrafish

The gene expression pattern of ADA-related genes was investigated in six different tissues of zebrafish. After dissection of brain, gills, heart, liver, skeletal muscle and kidney, the total RNA was isolated. Semi-quantitative RT-PCR experiments were performed and the relative abundance of *ADA1*, *ADAL*, *ADA2-1* and *ADA2-2* mRNA versus *β-actin* was determined (Table 2 and Fig. 3). The relative amount of *ADA1* transcripts was similar in all tissues studied (Fig. 3A). *ADAL* transcripts were more abundant in the liver and kidney, whereas a lower relative expression of this gene was detected in the heart (Fig. 3B). A product of 440 bp was obtained by RT-PCR, which corresponded to *ADA2-1*, and a 554 bp fragment, with distinct band

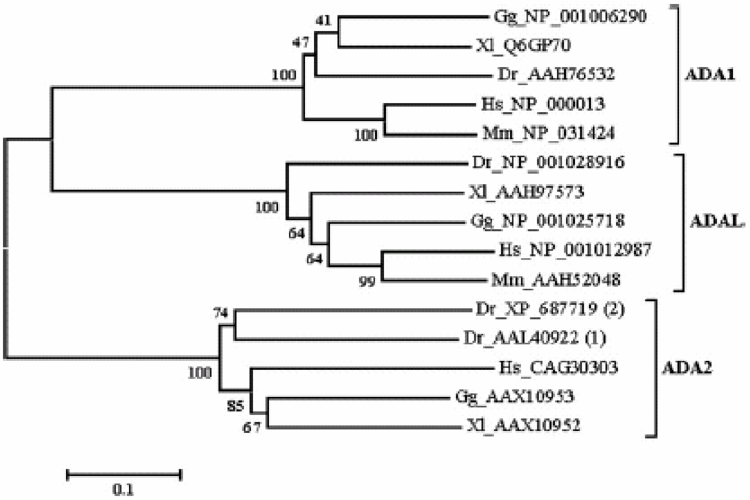


Fig. 1. Phylogenetic analysis of ADA-related family members. The deduced amino acid sequences were aligned with ClustalX program and the phylogenetic tree was constructed using Neighbor-Joining method, proportional (*p*) distance with MEGA 2.1 program. The phylogenetic tree grouped consistently (Dr) *Danio rerio*, (Xl) *Xenopus laevis*, (Gg) *Gallus gallus*, (Mm) *Mus musculus* and (Hs) *Homo sapiens* ADA1, ADAL and ADA2 orthologous sequences.

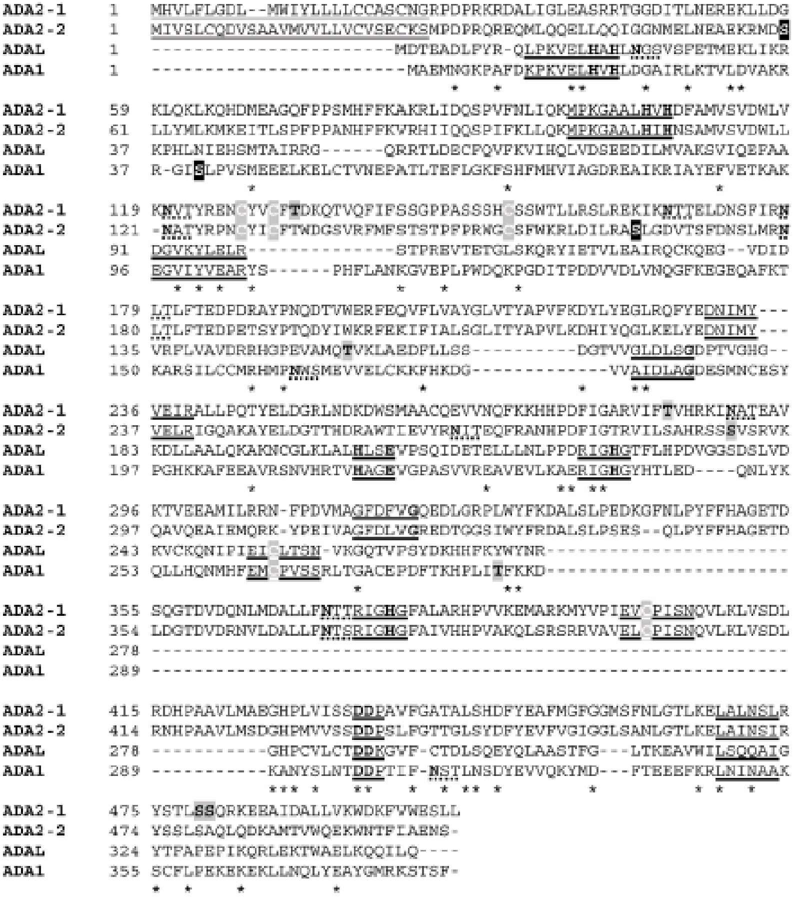


Fig. 2. Deduced amino acid sequences alignment of zebrafish ADA members. The eight conserved domains among the ADA subfamilies are underlined and the eight amino acids important for ADA activity in these domains are bold face. The signal peptide from the two ADA2 orthologous are double underlined, potential *N*-glycosylation sites are shown by dotted lines and the conserved cysteine residues from ADA1/ADAL and ADA2-1/ADA2-2 are indicated by white letters in gray boxes. Putative PKA phosphorylation sites are marked in black boxes, whereas PKC residues targets are demonstrated in gray boxes. The similarities among at least three different sequences are indicated by the asterisks.

intensities, was also amplified with the *ADA2-1* (Fig. 3C) even when PCR was carried out using high stringency conditions. The brain, gills and liver were the tissues that presented a higher expression of *ADA2-1*. A similar level of expression was evidenced in the muscle and kidney and this gene was less expressed in the heart. Although *ADA-2-2* gene expression could be detected only when the enhancer betain and Taq Platinum were added to PCR mixture, this gene had a similar expression pattern in all tissues studied (Fig. 3D).

ADA2-1 alternative splicing in zebrafish

In order to understand the significance of the 554 bp fragment, the nucleotide sequence of *ADA2-1* (AF384217) was

Table 2
Relative mRNA expression of ADA-related genes in zebrafish tissues

Genes	Optical densitometry (O.D.): ADA-related genes versus *β-actin* (mean±S.E.)					
	Brain	Gills	Heart	Liver	Muscle	Kidney
ADA1	0.70±0.08	0.65±0.02	0.69±0.02	0.67±0.01	0.71±0.04	0.60±0.10
ADAL	0.72±0.03	0.70±0.05	0.63±0.03^{d}	0.91±0.12^{c}	0.76±0.02	0.90±0.05
ADA2-1/T	1.02±0.06^{c,d,e,f}	0.92±0.04^{c,f}	0.52±0.06^{a,b}	0.63±0.07^{a}	0.68±0.08^{a}	0.51±0.09^{a,b}
ADA2-1	1.00±0.03^{c,e}	0.93±0.02^{c}	0.51±0.07^{a,b,d}	1.00±0.05^{c,e}	0.71±0.10^{a,d}	0.79±0.07
ADA2-2	0.85±0.08	0.93±0.04	0.89±0.003	0.86±0.06	0.83±0.03	0.87±0.07

The results were analyzed by ANOVA followed by Tukey HSD test as post-hoc, considering $P \leq 0.05$ as significant. The relative amount of mRNA levels was significantly different from [a]brain, [b]gills, [c]heart, [d]liver, [e]muscle and [f]kidney.

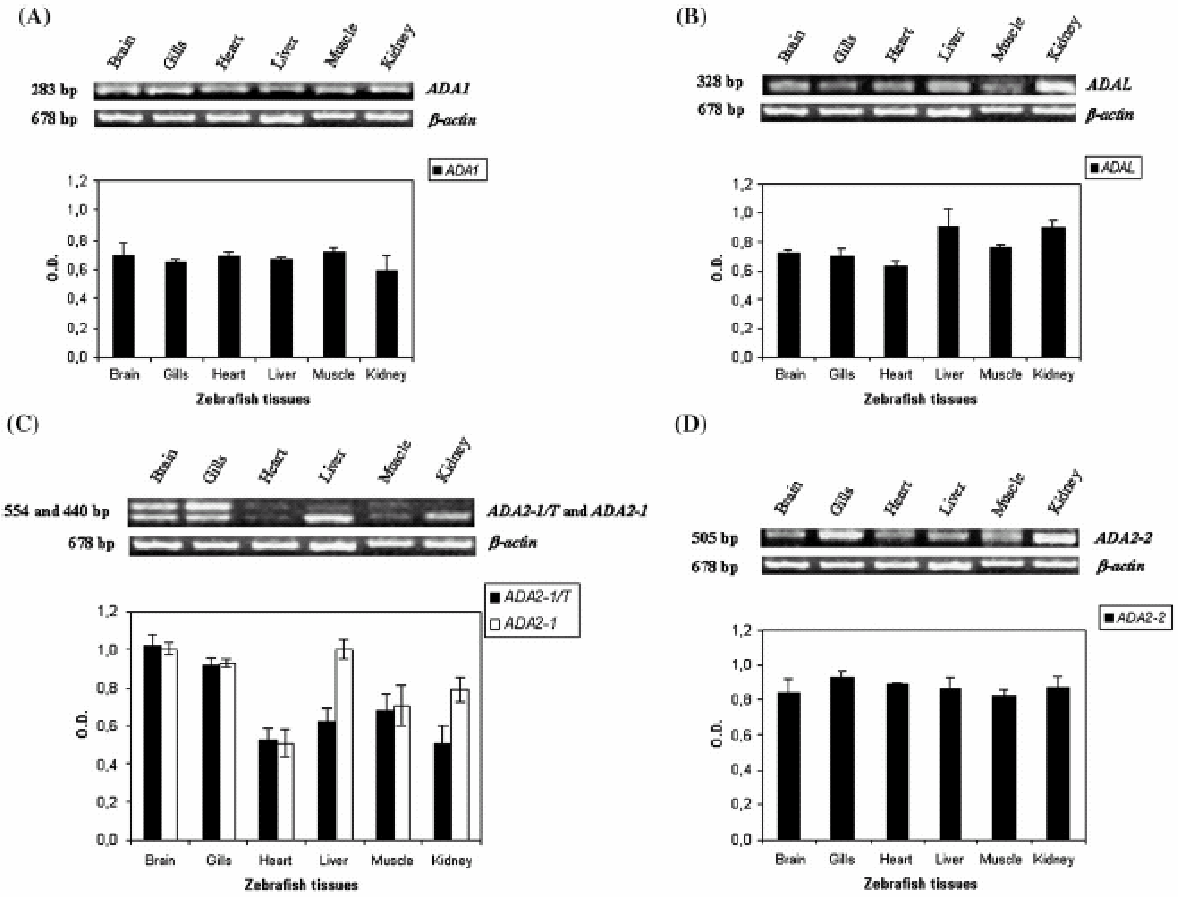


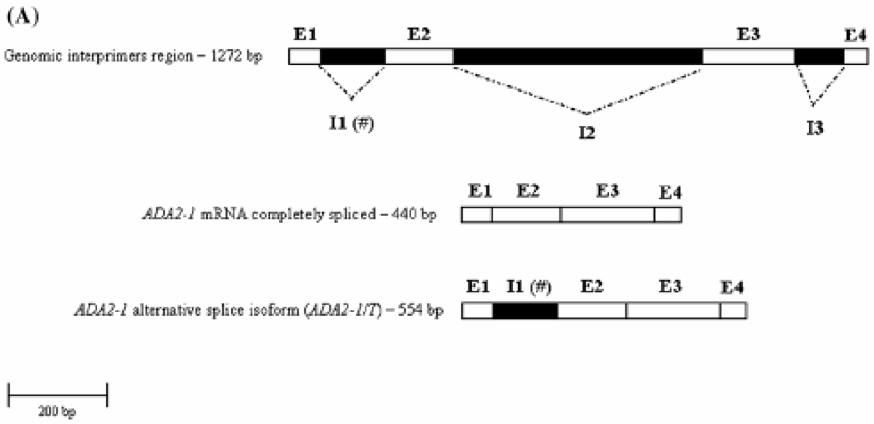
Fig. 3. Expression pattern of *ADA1* (A), *ADAL* (B), *ADA2-1* and *ADA2-1/T* (C) and *ADA2-2* (D) in zebrafish brain, gills, heart, liver, skeletal muscle and kidney. The amplifications resulted in a single product, except *ADA2-1*. The results were expressed as optical densitometry (O.D.) of the ADA-related genes versus *β-actin* expression (mean ±S.E.) of four independent replicate RT-PCR experiments.

compared with zebrafish genome and a high score with a genomic clone (BX004976) was observed. The comparison of interprimers region between the EST and genomic deduced amino acid sequences allowed the identification of four exons and three introns. The fragment length of the four exons was 440 bp, which was expected on *ADA2-1* gel analysis. However, the first intron of the interprimers sequence had a length of 114 bp indicating that the other fragment of 554 bp corresponded to a zebrafish *ADA2-1* alternative splicing (Fig. 4A). PCR products were sequenced and the results corroborated with the alternative splicing event. In addition, this isoform of *ADA2-1* in zebrafish is a transcript that is not completely translated, providing a truncated alternative splice isoform (*ADA2-1/T*) due to the appearance of a stop codon localized in the intron sequence (Fig. 4B). This transcript has only four of the eight ADA subfamily conserved domains and it is expressed differently than *ADA2-1* completely processed in the liver and kidney (Fig. 3C). The relative amount of mRNA levels of this gene was higher in the brain and gills. An intermediary expression pattern has been observed in the liver and muscle and a lower expression was identified in the heart and kidney.

Discussion

Adenosine deamination is an important mechanism able to control adenosine signaling in different tissues. In this study, a phylogenetic analysis confirmed the presence of distinct ADA-related genes in zebrafish. The deduced amino acid sequences alignment demonstrated the eight conserved domains among the different ADA enzymes with the catalytically important residues for ADA activity, which have already been demonstrated for these ADA members in several organisms (Maier et al., 2005). Furthermore, both zebrafish ADA2 orthologous sequences shared a signal peptide, demonstrating their potential role to cleave extracellular adenosine in zebrafish tissues. The alignment also demonstrated three conserved putative *N*-glycosylation sites for ADA2-1 and ADA2-2 and two ADA2-1 high score phosphorylation sites for Ser479 and Ser480 residues, suggesting that these regions might be important for posttranslational modifications.

The results demonstrated that *ADA1*, *ADA2* and *ADAL* are expressed in all tissues studied. Semi-quantitative RT-PCR assays have shown that the relative gene expression of *ADA1*


(A)
Genomic interprimers region – 1272 bp
E1
E2
E3
E4
I1 (#)
I2
I3
ADA2-1 mRNA completely spliced – 440 bp
E1
E2
E3
E4
ADA2-1 alternative splice isoform (ADA2-1/T) – 554 bp
E1
I1 (#)
E2
E3
E4
200 bp

(B)

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72792    AGT TTA CCT GAA GAC AAG GGT TTT AAC CTG CCC TAC TTC TTT CAC GCT
                      E   D   K   G   F   N   L   P   Y   F   F   H   A

72840    GGA GAG ACA ggt ttc ctt tga cgt ctc gct tct ttt atc tgc tac aaa
          G   E   T   G   F   L   *
```

Fig. 4. Alternative splice isoform of *ADA2-1* in zebrafish and the terminal region of *ADA2-1/T*. (A) The genomic BX004976 interprimers region with E (exons) and I (introns), the *ADA2-1* mRNA completely processed and the alternative splice isoform (*ADA2-1/T*) in zebrafish are showed. The intron not spliced in *ADA2-1/T* is represented by the symbol (#). (B) Nucleotide and deduced amino acid sequences from *ADA2-1/T*. The primer forward sequence is bold face and underlined, the nucleotides from intron sequence are represented in lower case and the stop codon is marked by an asterisk.

was similar in all tissues analyzed, whereas *ADAL* mRNA levels were more abundant in the liver and kidney and less expressed in the heart. The two ADA2 orthologous presented some interesting differences. While *ADA2-1* was easily detected in our experiments, being higher expressed in the brain, gills and liver, the *ADA2-2* products were obtained only when the RT-PCR mixtures were carried out with the enhancer betain. Therefore, even with a similar relative expression pattern of *ADA2-2*, it is plausible to hypothesize that this ADA2 form might be less expressed when compared to its paralogous *ADA2-1*. In relation to ADA1, the expression pattern in murine had already been determined in different tissues and elevated levels of the specific activity of this enzyme appeared to correlate directly with steady state levels of ADA mRNA (Chinsky et al., 1989). Thus, the presence of distinct ADA members and the differences in the relative amount of mRNA observed may suggest a distinct functional role for *ADA1*, *ADA2* and *ADAL* among zebrafish tissues.

The nucleoside adenosine is a molecule that plays several roles in different tissues. In CNS, adenosine acts as a neuromodulator, controlling the excitatory and inhibitory synapses (Fredholm et al., 2005). Evidence showed that adenosine contributes to insulin-stimulated muscle glucose transport by activating the A_1 receptor (Thong et al., 2007) and plays a role in muscle vasodilatation acting on extraluminal A_{2A} receptors (Marshall, 2007). Moreover, it mediates vasoconstriction of afferent arterioles through A_1 activation, increasing intracellular calcium concentration in mouse kidney (Hansen et al., 2007). It has been suggested that adenosine increases in the arterio-venous circulation in the gill during hypoxia, leading to an increase of blood supply to heart and gill tissue in *Hemiscyllium ocellatum* shark (Stenslokken et al., 2004). In the liver, it was demonstrated that this nucleoside reversibly inhibits Ca^{2+} fluxes and chemotaxis of hepatic stellate cells and upregulates TGF-β and collagen I mRNA, influencing the cell migration and differentiation at sites of injury (Hashmi et al., 2007). Considering the wide spectrum of actions induced by adenosine, it is possible to suggest that the transcriptional differences observed among ADA subfamilies in zebrafish could be important to regulate the adenosinergic signaling in each one of these tissues.

Until the present moment, there is no data about the cellular localization of ADAL and whether it participates in the adenosine catabolism. The lack of evidence about ADAL functionality in mammals does not signify that it is not physiologically important. In this sense, the existence of ADAL leads to the hypothesis that ADA function might be compartmentalized, spatially and temporally for various tissues (Maier et al., 2005). Although the adenosine deaminase activity of ADAL still remains unclear, we have demonstrated that *ADAL* had distinct levels of mRNA transcripts in zebrafish and thus, it is possible to suggest that this ADA member might be expressed and consequently play some physiological function in other organisms.

ADA2 has been identified as a novel family of growth factors (ADGFs) in various organisms including the mollusk *Aplysia californica* (Akalal et al., 2004) and *Drosophila melanogaster* (Maier et al., 2001). The extracellular adenosine deamination promoted by secreted ADA2 is required for their mitogenic function as growth factor (Zurovec et al., 2002). Previous study demonstrated the presence of six different ADGF genes in *Drosophila*, which were expressed differently during the developmental stages (Maier et al., 2001). Recent evidence showed the molecular characterization of *M. brassicae* ADGF and its role to stimulate cell proliferation by regulating the level of inosine (Zhang and Takeda, 2007). Furthermore, transgenic mice overexpressing *CECR1* adenosine deaminase in the heart presented high rate of phenotypic defects associated to embryonic and neonatal lethality (Riazi et al., 2005). The identification of two different ADA2 sequences (paralogous) was reported in zebrafish (Maier et al., 2005), but no studies were conducted so far. Our results have shown a lower mRNA transcript level of *ADA2-1* in the heart when compared to other tissues studied, which could be important for the cardiovascular homeostasis and wild-type phenotype. Although there is no evidence about ADA2 growth factor activity in zebrafish, the differences observed in *ADA2* expression could be important for the cell proliferation mediated by adenosine depletion among tissues.

In the present study, we obtained two different *ADA2-1* transcripts in zebrafish: an expected fragment of 440 bp, which corresponded to the mRNA completely spliced, and an alternative splice isoform with 554 bp. This fragment had the inclusion of the first intron of the genomic interprimers sequence and, interestingly, after deduced amino acids sequence analysis, it was observed that the *ADA2-1* isoform in zebrafish encodes a truncated protein (ADA2-1/T). The function of *ADA2-1/T* in zebrafish is uncertain. Since the deduced amino acids sequence of ADA2-1/T did not present four ADA conserved domains, which include the His238, Asp295 and Asp296 residues important for ADA activity, the adenosine deamination promoted by this enzyme could be altered. Considering that an alternative splicing was detected for *ADA2-1* gene, it is not possible to exclude the existence of other *ADA1*, *ADAL* and *ADA2-2* isoforms in zebrafish.

The process named alternative splicing is a tightly regulated post-transcriptional event, responsible to create a diverse array of mRNA from a single pre-mRNA (Lopez, 1998). The intron excision or retention is often differently regulated and may generate several mRNA variants, which encode distinct proteins (Wang et al., 2006). It was demonstrated that the “adenosine deaminase which acts on RNA” (ADAR) transcripts have distinct alternative splicing patterns in mammals and zebrafish (Slavov and Gardiner, 2002). Splicing variants may also result in truncated isoforms. For example, studies have shown that fibronectin (Liu et al., 2003) serine racemase (Konno, 2003), transposase (Gueguen et al., 2006) and TRKB receptors (Haapasalo et al., 2002) may be expressed as truncated isoforms, playing several biological functions. As truncated proteins seem to exert a complex regulatory effect, it is not possible, *a priori*, to determine whether ADA2-1/T is at least functionally active. However, the differential expression pattern of *ADA2-1/T* in zebrafish tissues leads to hypothesize that it may have some physiological importance in this vertebrate.

This is the first study to report a differential mRNA expression pattern of ADA-related genes in zebrafish. Like humans, zebrafish has three distinct members of ADA (ADA1, ADA2 and ADAL), with two different ADA2 forms (ADA2-1 and ADA2-2). Furthermore, the alternative splicing of *ADA2-1* translates to a truncated isoform suggesting that the adenosine deamination depends on the complexity of ADA-related enzyme activities associated with gene expression. Therefore, the differential expression pattern of ADA-related genes family suggests a fine-tuning regulation for adenosine deaminase activity in zebrafish adenosinergic system.

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CAPÍTULO 2

Denis Broock Rosemberg, Eduardo Pacheco Rico, Mario Roberto Senger, Renato Dutra Dias, Maurício Reis Bogo, Carla Denise Bonan, Diogo Onofre Souza.

Kinetic characterization of adenosine deaminase activity in zebrafish brain

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Kinetic characterization of adenosine deaminase activity in zebrafish brain

Denis Broock Rosemberg [1,*], Eduardo Pacheco Rico [1], Mario Roberto Senger [1], Renato Dutra Dias [2], Maurício Reis Bogo [3], Carla Denise Bonan [2], Diogo Onofre Souza [1]

[1] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul. Rua Ramiro Barcelos 2600-Anexo, 90035-003, Porto Alegre, RS, Brazil.

[2] Laboratório de Neuroquímica e Psicofarmacologia, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil.

[3] Laboratório de Biologia Genômica e Molecular, Departamento de Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900, Porto Alegre, RS, Brazil.

***Corresponding author:** dbrosemberg@gmail.com

Fax: +55-51-3308-5540

Abstract

Adenosine deaminase (ADA) activity is responsible for cleaving adenosine to inosine. In this study, we characterized the adenosine deamination in soluble and membrane fractions of zebrafish (*Danio rerio*) brain. The linearities of time and protein reactions were established and the temperature of 37ºC was carried out for the enzyme assays. A significant inhibition of 31.3% on adenosine deamination in membrane preparations was observed in the presence of 5 mM Zn^{2+}, which was prevented by the addition of 5 mM EDTA. For adenosine hydrolysis the optimum pH was in the range of 6.0–7.0 in soluble fraction and reached 5.0 in brain membranes. The apparent K_m values for adenosine deamination were 0.22 ± 0.03 (mean ± SEM, n=6) and 0.19 ± 0.04 mM (mean ± SEM, n=6) for soluble and membrane fractions, respectively. The apparent V_{max} value for soluble ADA activity was 12.3 ± 0.73 (mean ± SEM, n=6) nmol NH_3. min^{-1}. mg^{-1} of protein, whereas V_{max} value in brain membranes was 17.5 ± 0,51 (mean ± SEM, n=6) nmol NH_3. min^{-1}. mg^{-1} of protein. Adenosine and 2'-deoxyadenosine were the preferred substrates in both cellular fractions, whereas the guanine nucleosides were deaminated at lower rates. The effect of the inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was also studied. It was verified that 0.1 mM EHNA promoted a strong decrease on ADA activity in soluble fraction (82.3%) and caused a significant inhibition on adenosine deamination in brain membranes (26.5%). The presence of soluble and membrane-bound ADA activities in zebrafish brain may be important to regulate the ratio of adenosine/inosine nucleosides in both intracellular and extracellular millieu in the CNS of this species.

Key words: adenosine; inosine; adenosine deaminase; zebrafish; brain

1. Introduction

Adenosine is an important signaling molecule within tissues. This nucleoside can be released depending on the intracellular concentrations, or can be rapidly formed from extracellular ATP breakdown due to ecto-nucleotidase activities [1]. The effects elicited by adenosine are mediated by metabotropic P1 receptors, which include the negatively coupled to adenylate cyclase A_1 and A_3 receptors and the positively coupled to adenylate cyclase A_{2A} and A_{2B} receptors [2, 3].

In the central nervous system (CNS), adenosine acts as a neuromodulator, controlling both inhibitory and excitatory synapses [3]. Despite the neuroprotective roles promoted by extracellular adenosine, the activation of A_{2A} receptors may aggravate tissue damage [4]. Therefore, the interaction of adenosine with its respective purinoreceptors must be tightly regulated. The control of the adenosinergic signaling can be exerted by adenosine uptake via bi-directional transporters, followed by intracellular phosphorylation to AMP by adenosine kinase (AK) or deamination to inosine by adenosine deaminase (ADA) [5]. Furthermore, studies have shown that the extracellular concentrations of adenosine may also be regulated by ecto-ADA activity [6, 7].

A phylogenetic study demonstrated that the adenyl-deaminase family includes distinct ADA subfamilies [8]. Almost all human ADA activity has been attributed to ADA1, which plays an important role in protecting T cells from an adenosine-mediated inhibition of proliferation [9, 10]. The severe combined immunodeficiency (SCID) is a consequence of ADA1 dysfunction in childhood, causing severe tissues damage [11]. Despite its intracellular activity, ADA1 may be a membrane-bound protein, acting as an ecto-ADA [12]. Studies demonstrated that this enzyme may be co-localized with CD26 (DPPIV - dipeptidyl-peptidase IV), A_1 and A_{2B} receptors in neuronal and non-neuronal cells, suggesting a possible involvement of ecto-ADA to regulate adenosine signaling via P1 receptors [6, 13, 14].

The other ADA subfamilies are constituted by ADA2 and adenosine deaminase-like (ADAL). The deduced amino acid sequence analysis of ADA2 revealed that it is encoded by cat-eye syndrome critical region candidate 1 (*CECR1*) gene in vertebrates [9]. Furthermore, it was verified that human plasma ADA2 may be secreted by activated monocytes [15] and its activity has been suggested to regulate cell proliferation as a new family of growth factors, named adenosine deaminase-related growth factors (ADGFs) [16]. In contrast to ADA2, there is no much evidence about ADAL functionality so far. However, this ADA member presents conserved catalytically important sites, which may be important for adenosine deamination [8].

Zebrafish (*Danio rerio*) is a small freshwater teleost widely used in genetic and biochemical studies that combines the relevance of a vertebrate with the scalability of an invertebrate [17, 18]. It has already been shown that zebrafish genes are highly conserved, since its genome presents a syntenic relationship with the human genome [19]. Concerning the purinergic system, studies from our laboratory reported the presence of ecto-nucleotidase activities in zebrafish brain membranes [20, 21] and the differential expression pattern of distinct ADA subfamilies in zebrafish tissues [22].

Considering the putative relevance of purinergic signaling in zebrafish brain [23, 24] and that adenosine deamination represents a mechanism able to regulate intracellular and extracellular levels of adenosine, the goal of this study was to perform a kinetic characterization of ADA activity in soluble and membrane fractions obtained from zebrafish brain.

2. Materials and methods

2.1. Chemicals

Adenosine, 2'-deoxyadenosine, guanosine, 2'-deoxyguanosine, EGTA, EDTA, erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochoride (EHNA), Coomassie Blue G and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenol, sodium nitroprusside and magnesium chloride were purchased from Merck (Darmstadt, Germany). All other reagents used were from high analytical grade.

2.2. Fish maintenance

Adult wild-type zebrafish (*Danio rerio*), around 3-6 month-old, were obtained from commercial supplier (Delphis, RS) and acclimated for at least 2 weeks in a 50-L thermostated aquarium filled with continuously aerated unchlorinated water. The temperature was kept between 26 ± 2ºC under a 12-h light-dark controlled photoperiod and the animals were fed with commercial fish pellet twice a day. The use and maintenance of zebrafish were according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.3. Preparation of soluble and membrane fractions

In order to obtain both cellular fractions, zebrafish were euthanized and whole brains were initially homogenized in 20 volumes (v/w) of chilled phosphate buffer saline (PBS), with 2 mM EDTA, 2 mM EGTA, pH 7.4 in a glass-Teflon homogenizer. The preparation of brain membranes was according to the method described previously [25]. In brief, the homogenate was centrifuged at 800 *g* for 10 min and the supernatant fraction was subsequently centrifuged for 25 min at 40 000 *g*. The resultant supernatant and the pellet obtained corresponded to the soluble and membrane fractions, respectively. The supernatant was collected and kept on ice for enzyme assays. The pellet was frozen in liquid nitrogen, thawed, resuspended in PBS and centrifuged for 20 min at 40 000 *g*. This fresh-thaw-wash procedure was used to ensure the lysis of the membrane vesicles. The final pellet was resuspended and used for biochemical assays. The material was maintained at 2-4°C throughout preparation.

2.4. Adenosine deaminase assay

Adenosine deaminase activity was determined spectrophotometrically by measuring the ammonia produced over a fixed time using a Berthelot reaction as previously reported [26]. After the preparation of soluble and membrane fractions, the optimum conditions for adenosine hydrolysis were determined. The brain fractions (5–10 μg protein) were added to the reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 50 mM sodium acetate buffer (pH 5.0) for the experiments with soluble and membrane fractions, respectively, in a final volume of 200 μl. The samples were pre-incubated for 10 min at 37ºC and the reaction was initiated by the addition of substrate (adenosine or other, as indicated) to a final concentration of 1.5 mM (with exception for substrate curves). After incubated for 75 min (soluble fraction) and 120 min (membranes) (with exception for substrate curves), the reaction was stopped by adding the samples on a 500 μl of phenol-nitroprusside reagent (50.4 mg of phenol and 0.4 mg of sodium nitroprusside/ml). Controls with the addition of the enzyme preparation after mixing with phenol-nitroprusside reagent were used to correct non-enzymatic hydrolysis of substrates. The reaction mixtures were immediately mixed to 500 μl of alkaline-hypochlorite reagent (sodium hypochlorite to 0.125% available chlorine, in 0.6 M NaOH) and vortexed. Samples were incubated at 37ºC for 15 min and the colorimetric assay was carried out at 635 nm. Incubation times and protein concentrations were chosen in order to ensure the linearity of the reactions. Specific activity was expressed as nmol of NH_3. min^{-1}. mg^{-1} of protein.

2.5. Characterization of ADA activity

To verify the influence of temperature, the enzyme assays were carried out under standard assay conditions at various temperatures in the range 25–60ºC. The optimum pH for enzyme activity was assayed with 50 mM buffers sodium acetate (pH 3.5–6.0), sodium phosphate (pH 6.5–7.4) and sodium carbonate bicarbonate buffer (pH 8.0–9.0). The effect of divalent cations (Ca^{2+}, Mg^{2+} and Zn^{2+}) on ADA activity was tested after preincubation with metals for 10 min. The apparent K_m and V_{max} values for adenosine deamination on soluble and membrane fractions were determined from Eadie-Hofstee plots by using substrate concentrations from 0.10 to 3.0 mM.

2.6. Protein determination

Protein was measured by the Coomassie blue method [27] using bovine serum albumin as a standard.

2.7. Statistical analysis

All experiments were carried out in replicate and means ± SEM were presented. Data were analyzed by Student's t-test or one-way analysis of variance (ANOVA), followed by Tukey test as post-hoc. *P* values ≤ 0.05 were considered as significant.

3. Results

3.1. Adenosine deamination as a function of time and protein concentration

We evaluated the adenosine deamination in zebrafish brain as a function of time and protein concentration (1.5 mM adenosine as initial substrate) in order to determine the correct assay conditions. The soluble and membrane fractions were incubated as described in Materials and Methods. The deamination promoted by ADA activity was linear up to 105 min (soluble - Fig. 1A) or up to 180 min (membrane-bound - Fig. 1B). As the ADA activity during 75 min of incubation (soluble fraction - Fig. 2A) and 120 min (membranes - Fig. 2B) was linear in the range of 5 –20 µg, we chose to use 5–10 µg of protein from both fractions in the further enzyme assays.

3.2. Influence of temperature and pH dependence

To evaluate the influence of temperature on ADA activity, the enzyme assays were carried out in a range of 25–60ºC after a 10 min of pre-incubation period. The specific activity increased up to 45ºC (data not shown); thus, the temperature of 37ºC was chosen for further assays in order to lessen the risk of protein denaturation in both cellular fractions. This same incubation temperature was previously used in our laboratory for characterization of ATP, ADP and AMP hydrolysis in zebrafish brain membranes [20, 21].

To verify the pH dependence, the effect of different pH was examined in a range between 5.0–8.0 and 3.5–9.0 for soluble and membrane fractions, respectively. The buffers used were 50 mM sodium acetate (applied in a pH range from 3.5–6.0), sodium phosphate (used in a pH range from 6.5–7.4) and sodium carbonate bicarbonate buffer (assayed from pH 8.0–9.0), as previosly described [28]. The results have shown that the optimum pH for soluble ADA was in a range between 6.0–7.0, (Fig. 3A) whereas the optimum pH for membrane-bound ADA was 5.0 (Fig. 3B). Therefore, the pH 7.0 and 5.0 were chosen for the subsequent experiments with soluble and membrane-bound ADA, respectively.

3.3. Effect of divalent cations on ADA activity

In order to investigate a possible effect of divalent cations on ADA activity, 5 mM Ca^{2+}, Mg^{2+} and Zn^{2+} were used (*n*=5). Zn^{+2} was the only cation able to decrease (by 31 %) the ADA activity, specifically from brain membranes, which was prevented by the addition of 5 mM EDTA (Fig. 4). The soluble ADA activity was not altered in the presence of these metals (data not shown).

3.4. Kinetic parameters of zebrafish brain ADA

The adenosine deamination was determined at adenosine concentrations ranging from 0.15 to 3.0 mM in soluble (Fig. 5A) and 0.10 to 3.0 mM in membranes (Fig. 5B) fractions. The apparent Michaelis-Menten constants (K_m app) and maximum velocities (V_{max} app) were estimated from Eaddie-Hofstee plots (Fig. 5). The apparent K_m were 0.22 $\pm$ 0.03 mM (mean $\pm$ SEM, *n*=6) for soluble and 0.19 $\pm$ 0.04 mM (mean $\pm$ SEM, *n*=6) for brain membrane preparations. The calculated V_{max} in soluble and membrane fractions were 12.30 $\pm$ 0.73 (mean $\pm$ SEM, *n*=6) and 17.50 $\pm$ 0.51 (mean $\pm$ SEM, *n*=6) nmol $NH_3.min^{-1}.mg^{-1}$ of protein, respectively. The insets show the saturation curves.

3.5. Different nucleoside specificity

The relative substrate specificity of zebrafish brain ADA was determined (Table 1). Adenosine and 2'-deoxyadenosine were the most preferred substrates, compared to guanosine 2'-deoxyguanosine. The soluble fraction demonstrated a preference for 2'-deoxyadenosine over adenosine, whereas the membrane fraction demonstrated a preference for adenosine. The specific activity was higher in the membrane preparation for adenosine (20.9 ± 1.3, mean ± SEM, *n*=5) and for 2'-deoxyadenosine (23.6 ± 1.5, mean ± SEM, *n*=5) in the soluble fraction.

3.6. Inhibition of ADA activity by EHNA

Several studies have demonstrated that EHNA is a potent inhibitor of ADA1 activity [29, 30, 31]. Thus, we measured the adenosine deamination in zebrafish brain in the presence and in the absence of 0.1 mM EHNA (Table 2). Although the ADA activities in both soluble and membrane fractions were inhibited by this compound, the inhibition was more pronounced over the soluble (82.3%), than over the membrane-bound (26.5%) ADA activity.

4. Discussion

In the present study, we evaluated the biochemical properties of ADA activity in soluble and membrane fractions of zebrafish brain. Previous study showed that there are different ADA subfamilies, such as ADA1, ADA2 and ADAL, grouped in the adenyl deaminase family [8]. The transcriptional profile of these ADA members was already reported, demonstrating that *ADA1*, two paralogous of *ADA2* (*ADA2-1* and *ADA2-2*), a truncated *ADA2-1* alternative splice isoform (ADA2-1/T) and *ADAL* are expressed in zebrafish brain [22]. Therefore, it is possible to suggest that the presence of these enzymes may be contributing in a different manner for the adenosine levels in distinct cellular fractions.

In order to verify whether adenosine deamination in zebrafish brain may be altered in the presence of a classical inhibitor of ADA1, both cellular fractions were incubated in the presence and in the absence of 0.1 mM EHNA. While the soluble ADA activity displayed an inhibitory effect of 82.3%, the adenosine hydrolysis in membrane fractions decreased 26.5%. It was suggested that ADA1 typically constitutes 90–100% of the total intracellular ADA activity [29], which lead us to hypothesize that this enzyme probably is the major responsible for adenosine deamination in the soluble fraction of zebrafish brain. However, if ADAL is able to promote the adenosine deamination, the effect of EHNA on its enzyme activity still remains to be investigated, since its deduced amino acid sequence shares similarities with the classical ADA1 members [8, 22].

The substrate curve demonstrated that the apparent K_m for adenosine calculated by linear regression were around 0.2 mM in zebrafish brain. The estimated V_{max} for adenosine deamination in soluble and membrane fractions were 12.3 and 17.5 nmol $NH_3.min^{-1}.mg^{-1}$ of protein, respectively. The kinetic data obtained in this study are in accordance with other studies related to ADA activity. The characterization of an ecto-ADA from porcine cerebral cortex synaptosomes reported that its K_m for adenosine was in the range of 0.29 mM [7]. Studies also demonstrated that there are some variations of K_m among different ADA members. The K_m value of *Hyalomma dromedarii* ADA2 was estimated to 0.5 mM adenosine [28], which is relatively close to several adenosine deaminases from different sources, such as rat brain (0.45 mM) [32], bovine brain (0.4 mM) [33], human (0.46 mM) and chicken liver (0.33 mM) [29]. However, lower K_m

values were reported for ADA activity from mice intestine (0.023 mM) [34] and from the sand fly *Lutzomyia longipalepis* (0.01 mM) [35]. Despite the high K_m observed for adenosine in consider to its physiological concentrations (which apparently does not exceed 30 μM), it was already demonstrated that ADA is necessary for an efficient coupling of purinoreceptors A_1 with the signal transduction machinery [36]. In addition, studies have shown that the efficiency of A_1 adenosine receptors signaling may be enhanced by ADA inhibition [37, 38]. Thus, the presence of a membrane-bound ADA in zebrafish brain may also play an extra-enzymatic role in the molecular adhesion and/or even in non-enzymatic regulation of adenosine receptors activity in physiological conditions.

The influence of pH on the denosine deamination in zebrafish brain was verified and the results demonstrated that the optimal pH in the soluble and membrane fractions for ADA activity reached at 7.0 and 5.0, respectively. Previous study demonstrated that the pH dependence for the reaction catalyzed by intestinal ADA of mice displayed a bell shaped curve, with a decreased about 50% of the activity below pH 5.0 on acidic side and above pH 9.0 on the basic side [34]. Although the purified human and chicken liver ADA1 had shown the optimal activity in the range of pH 7.4 [29], it was verified that the ecto-ADA from porcine brain had a very broad optimum pH, in the range 6.0–9.0 [7]. Despite the highest ADA1 activity at pH 7.4, the human ADA2 reaches maximal activity at more acidic pH [9]. Although there is no evidence about the optimal pH and functionality of ADAL so far, it was demonstrated that this member presents important amino acid residues for adenosine deamination [8, 22]. Considering that it is not possible to rule out the contribution of different ADA members for adenosine cleavage in membrane fractions, it is plausible to hypothesize that the higher ADA activity at pH 5.0

could be a result of a combination of different enzyme activities. Moreover, the preference of ADA for acidic pH in zebrafish brain membranes could be physiologically important during stress, hypoxia or brain injury, when adenosine levels are significantly elevated and the pH decreases in the extracellular fluid [39, 9, 40].

The adenosine deamination in zebrafish brain was also evaluated in the presence of divalent cations, such as Ca^{+2}, Mg^{+2} and Zn^{+2}. The results have shown that only the exposure to 5 mM Zn^{+2} was able to decrease the ADA activity in the membrane fraction. The effect of metals on ADA activity still remains controversial. Previous data showed that zinc may play a critical role for adenosine deamination being located deep within the substrate binding cleft in a tetrahedral geometry [41, 42]. Nevertheless, it was also demonstrated that zinc and other divalent cations are able to interact with other amino acid residues and induce an inhibition of the enzyme activity [41]. The characterization of ADA2 from camel tick showed that 5 mM Mg^{2+} and Zn^{2+} caused a significant decrease of its activity [28]. Considering that 5 mM EDTA plus Zn^{2+} prevented the inhibitory effect of zinc, our results reinforce the idea that this metal may act as an inhibitor of ADA activity. The presence of EDTA in the assay solution for adenosine deamination did not promote significant changes on enzyme activity during the time course of the assay, indicating that this molecule does not alter the nucleoside binding site or affect protein structure. Since it had been shown that a probable bound metal cofactor is inaccessible to chelators in a high affinity site [41], we conclude that 5 mM EDTA was able to prevent an inhibitory effect of Zn^{2+} associated at sites readily accessible to the solvent.

The preference to adenine over guanine nucleosides was demonstrated for both soluble and membrane fractions. While the soluble ADA displayed a strong preference for 2'-deoxyadenosine as substrate, the membrane-bound ADA preferred adenosine over

2'-deoxyadenosine. In the literature, it was already demonstrated that the preference for both adenine nucleosides may be varied and that adenosine and 2'-deoxyadenosine are the classical substrates for adenosine deaminase [29, 30]. On the other hand, the guanine nucleosides, guanosine and 2'-deoxyguanosine, were deaminated at considerable lower rates than adenine nucleosides in both cellular fractions.

This study describes some enzymatic and kinetic properties of adenosine deamination in both soluble and membrane preparations from zebrafish brain, which displayed a strong preference for adenine over guanine nucleosides. The presence of ADA activity in the brain membranes, suggests the presence of an ecto-ADA in zebrafish. Considering that previous study demonstrated the co-localization of ADA, CD26 and A_1 receptors in goldfish brain [43], it is interesting to hypothesize that they may also be co-localized in zebrafish brain. In conclusion, the adenosine deamination in the CNS of zebrafish may be a key component for controlling the adenosine levels in both intracellular and extracellular milieu. These data may be important to clarify the mechanisms of adenosine-mediated cell signaling inactivation in zebrafish, which could lead to better understanding the role of the purinergic system in this species.

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Figure legends

Fig. 1: Time course for adenosine deamination by soluble (A) and membrane (B) fractions from zebrafish brain. Data represent means ± SEM of four different replicate experiments.

Fig. 2: Effect of increasing protein concentrations on ADA activity of soluble (A) and membrane (B) fractions from zebrafish brain. Data represent means ± SEM of four different replicate experiments.

Fig. 3: pH dependence of adenosine hydrolysis by soluble (A) and membrane (B) preparations from zebrafish brain. The enzyme activity was determined as described in Material and Methods using 50 mM of the following buffers: sodium acetate (pH 3.5–6.0), sodium phosphate (pH 6.5–7.4) and sodium carbonate bicarbonate buffer (pH 8.0–9.0). Data represent means ± SEM of four different replicate experiments.

Fig. 4: Effect of divalent cations on ADA activity from membrane preparations of zebrafish brain. Data represents means ± SEM of four different replicate experiments. The asterisk represents a significant difference from control group (ANOVA, followed by Tukey test as post-hoc, $p<0.05$).

Fig. 5: Eadie-Hofstee plots for adenosine deamination by soluble (A) and membrane (B) fractions of zebrafish brain. The results were obtained with a nucleoside concentration ranging from 0.15 to 3.0 mM for soluble and 0.10 to 3.0 mM for membrane preparations.

The insets show the enzyme activity for both fractions and the best-fit analysis indicated a linear relationship. Six independent experiments were performed, with entirely consistent results. Data represents a typical experiment.

Abbreviations: ADA, adenosine deaminase; ADGF, adenosine deaminase-related growth factor; *CECR1*, cat eye critical region candidate gene 1; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine.

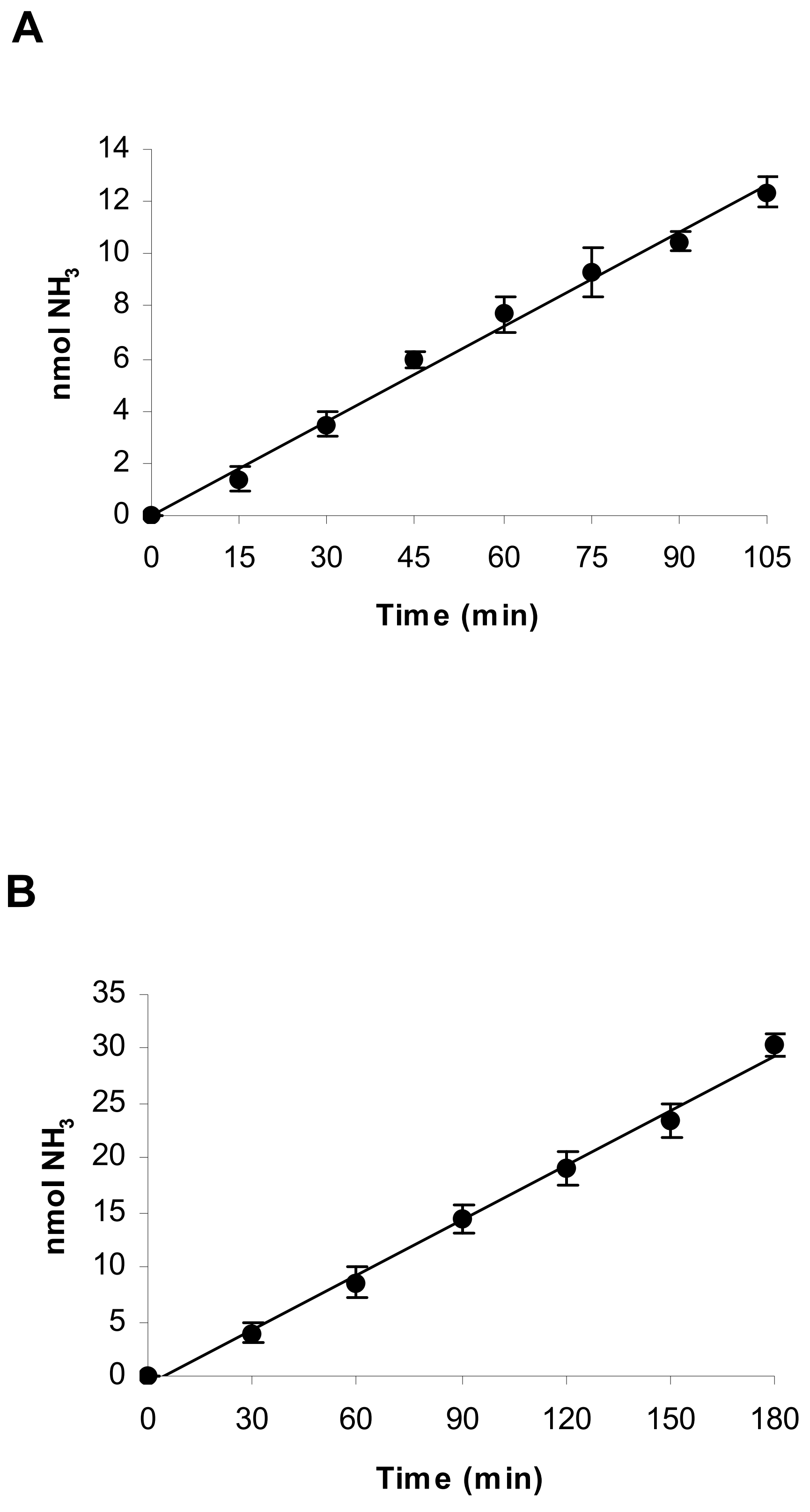
A
nmol NH_3
14
12
10
8
6
4
2
0
0
15
30
45
60
75
90
105
Time (min)
B
nmol NH_3
35
30
25
20
15
10
5
0
0
30
60
90
120
150
180
Time (min)

Fig. 1

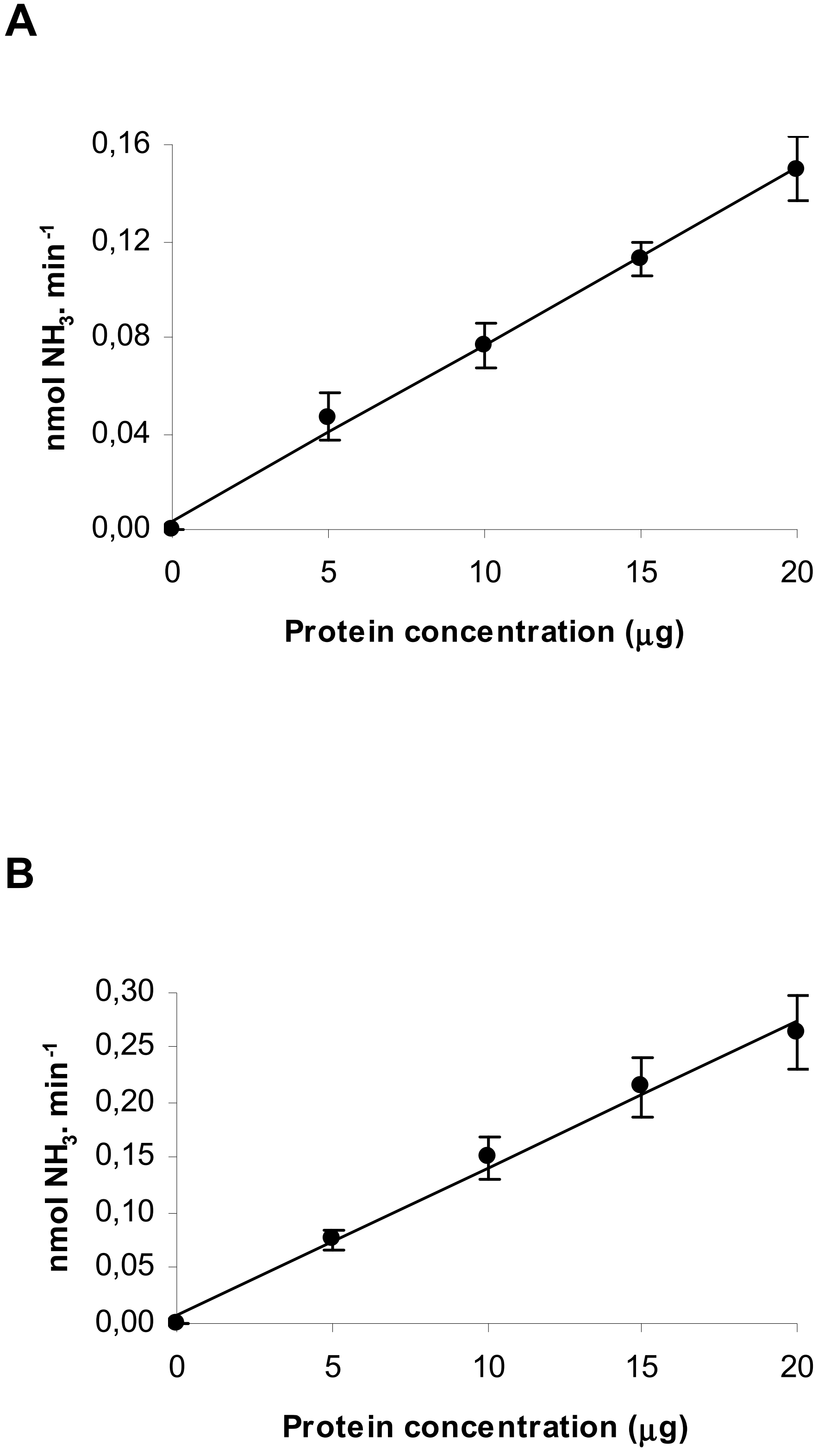
A
0,16
0,12
0,08
0,04
0,00
nmol NH$_3$. min^{-1}
0
5
10
15
20
Protein concentration (μg)
B
0,30
0,25
0,20
0,15
0,10
0,05
0,00
nmol NH$_3$. min^{-1}
0
5
10
15
20
Protein concentration (μg)

Fig. 2

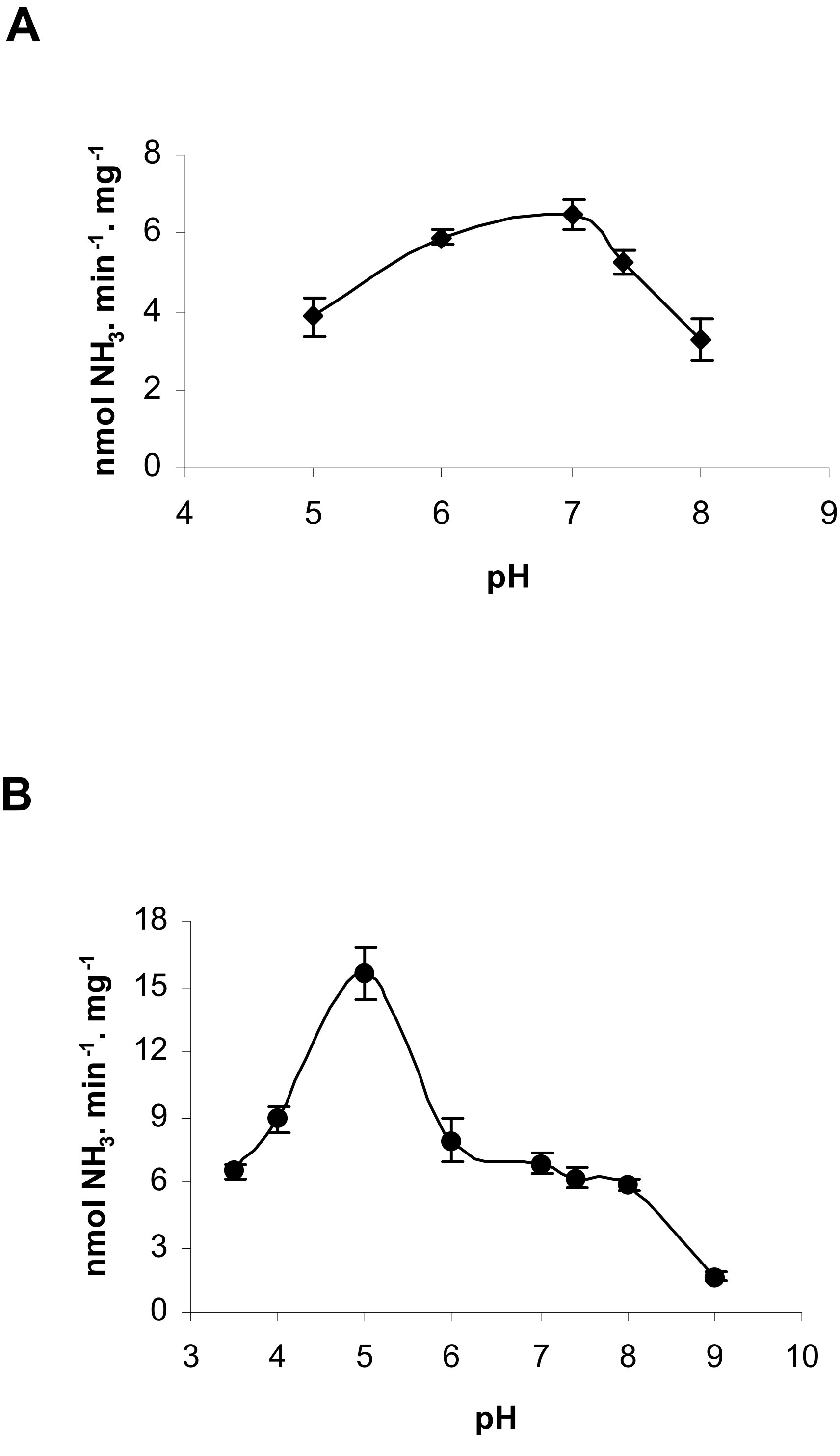
A
nmol NH_3. min^{-1}. mg^{-1}
8
6
4
2
0
4
5
6
7
8
9
pH
B
nmol NH_3. min^{-1}. mg^{-1}
18
15
12
9
6
3
0
3
4
5
6
7
8
9
10
pH

Fig. 3

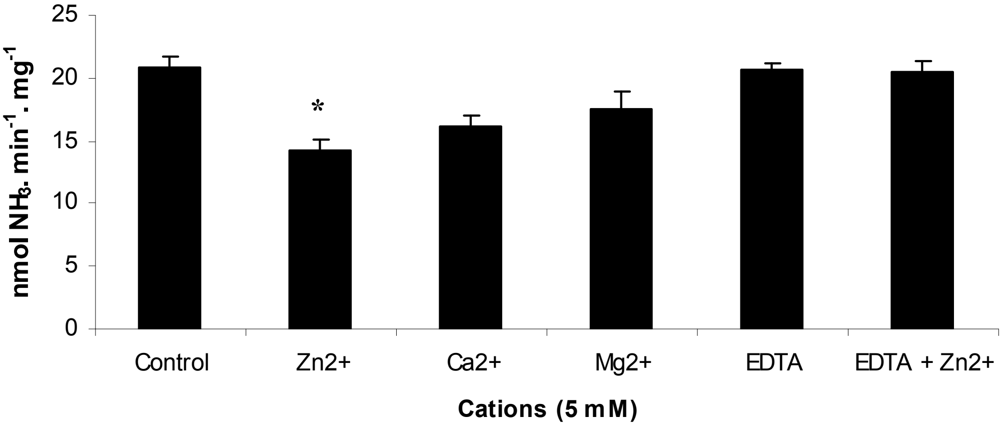
nmol NH$_3$. min^{-1}. mg^{-1}
25
20
15
10
5
0
*
Control
Zn2+
Ca2+
Mg2+
EDTA
EDTA + Zn2+
Cations (5 mM)

Fig. 4

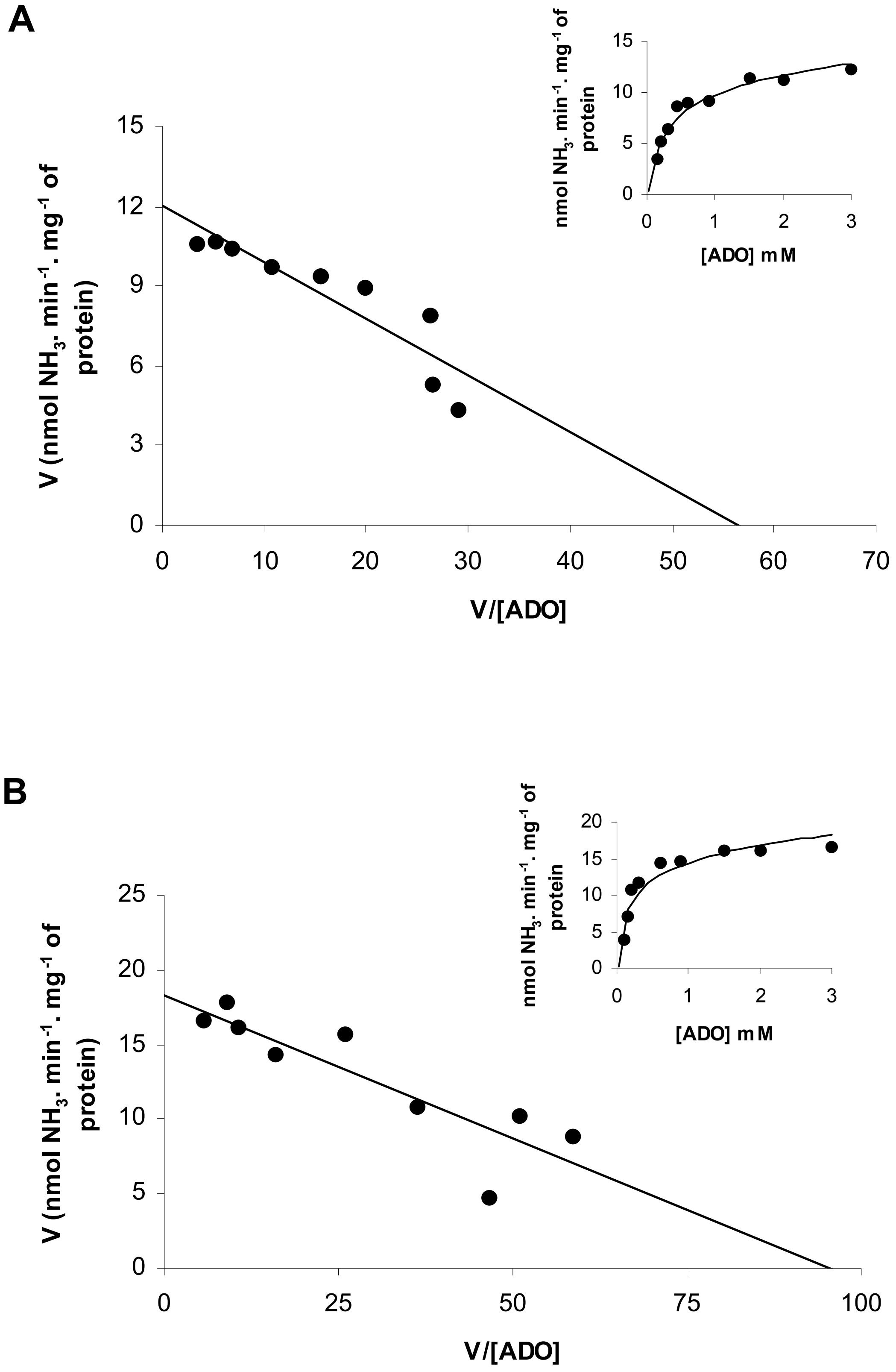
A
V (nmol NH$_3$. min^{-1}. mg^{-1} of protein)
0
3
6
9
12
15
0
10
20
30
40
50
60
70
V/[ADO]
nmol NH$_3$. min^{-1}. mg^{-1} of protein
0
5
10
15
0
1
2
3
[ADO] mM
B
V (nmol NH$_3$. min^{-1}. mg^{-1} of protein)
0
5
10
15
20
25
0
25
50
75
100
V/[ADO]
nmol NH$_3$. min^{-1}. mg^{-1} of protein
0
5
10
15
20
0
1
2
3
[ADO] mM

Fig. 5

Table 1: Relative substrates specificity of zebrafish brain ADA.

Substrate (1.5 mM)	**Specific activity (mean ± SEM)**		**Relative activity (%)**	
	Soluble	Membrane	Soluble	Membrane
Adenosine	11.2 ± 0.05 **	20.9 ± 1.3 **	100	100
2'-Deoxyadenosine	23.6 ± 1.5 *	5.4 ± 0.8 *	210.7	25.9
Guanosine	1.8 ± 0.2 *,**	1.7 ± 0.2 *,**	16.1	8.1
2'-Deoxyguanosine	2.0 ± 0.2 *,**	1.8 ± 0.3 *,**	17.9	8.6

* Significantly different from adenosine (ANOVA, followed by Tukey test as post-hoc, $p<0.05$, *n*=5).

** Significantly different from 2’-deoxyadenosine (ANOVA, followed by Tukey test as post-hoc, $p<0.05$, *n*=5).

Table 2: Inhibition of zebrafish brain ADA by EHNA.

Group	**Specific activity (mean ± SEM)**		**Relative activity (%)**	
	Soluble	Membrane	Soluble	Membrane
Control	11.3 ± 1.1	23.1 ± 0.8	100	100
EHNA	2.0 ± 0.2 *	16.9 ± 1.0 *	17.7	73.5

* Significantly different from control group (Student’s t-test, $p<0.05$, *n*=4).

PARTE III

III.1. Discussão

III.1.1. Considerações gerais

Os resultados apresentados neste estudo possibilitaram a identificação de distintos genes relacionados à ADA em zebrafish. Diferentes subfamílias pertencentes à família das adenil-deaminases, tais como ADA1, ADA2 e ADAL foram identificadas em zebrafish utilizando genes ortólogos de humano e rato para a busca em bancos genômicos. Desta forma, foi possível a confirmação da existência de dois genes parálogos da ADA2 (*ADA2-1* e *ADA2-2*). Além disso, todos os membros identificados são expressos em zebrafish e podem estar contribuindo para a atividade de desaminação de ADO em frações solúvel e de membrana no cérebro deste vertebrado.

III.1.2. Mapeamento do padrão de expressão dos genes relacionados à ADA em zebrafish

As seqüências dos genes relacionados à ADA em zebrafish obtidas a partir da busca em bancos genômicos foram analisadas através de uma análise filogenética, a qual agrupou consistentemente os diferentes membros dentro das suas respectivas subfamílias com um alto valor de suporte.

Este estudo possibilitou a confirmação da existência de regiões altamente conservadas e a presença de peptídeo sinal para os ortólogos da ADA2, como previamente demonstrado (MAIER et al., 2005). Além disso, os altos escores de sítios putativos de *N*-glicosilação e fosforilação obtidos demonstram que os membros relacionados à ADA podem sofrer modificações pós-traducionais, cujos efeitos na atividade enzimática ainda necessitam ser elucidados.

Para a análise do padrão transcricional, primers específicos para cada um dos membros relacionados à ADA foram desenhados e experimentos de RT-PCR semi-quantitativos foram conduzidos. Em virtude da β-actina apresentar um padrão de expressão similar nos tecidos, esta foi empregada como gene de expressão constitutiva para a normatização dos experimentos de RT-PCR. Os resultados demonstraram um padrão de expressão tecidual distinto dos genes *ADA1*, *ADA2-1*, *ADA2-2* e *ADAL* em cérebro, brânquias, coração, fígado, músculo esquelético e rim de zebrafish. Além disso, a estratégia possibilitou a identificação de uma isoforma truncada de splicing alternativo da *ADA2-1* (*ADA2-1/T*), cujos níveis relativos de expressão variam de acordo com o tecido.

As diferentes funções da ADO nos tecidos tem sido bastante estudadas. Na fenda sináptica, a ligação deste nucleosídeo em purinoreceptores P1 exerce um papel na neuromodulação (FREDHOLM et al., 2005). Estudos relacionados ao transporte de glicose estimulado por insulina no músculo têm demonstrado que a adenosina possui um importante papel na ativação de receptores A_1 (THONG et al., 2007). Outra função conhecida deste nucleosídeo no tecido muscular é a vasodilatação devido a sua interação com receptores A_{2A} extra-luminais (MARSHALL, 2007). Além disso, a ativação de receptores A_1 em arteríolas aferentes leva a um aumento da concentração de cálcio intracelular em rins de camundongos (HANSEN et al., 2007). Nas brânquias e no coração do tubarão *Hemiscyllium ocellatum*, um aumento dos níveis de adenosina em situações de hipóxia é capaz de desencadear um aumento do suprimento sangüíneo nestes tecidos (STENSLOKKEN et al., 2004). Também foi demonstrado que, no fígado, a ADO pode exercer efeitos sobre a migração e diferenciação celular em áreas lesionadas (HASHMI et al., 2007). Devido ao amplo espectro de ações exercidas pela ADO em diversos tecidos, as diferenças no padrão transcricional dos genes relacionados à ADA podem ser importantes para o controle da sinalização adenosinérgica nestes tecidos.

Na literatura, a identificação de uma seqüência deduzida de aminoácidos similar às adenosinas deaminases clássicas (ADA1 e ADA2) já tinha sido previamente descrita, sendo este novo membro denominado ADAL (MAIER et al., 2005). Esta outra subfamília também foi agrupada dentro da família das adenil-deaminases e a presença de resíduos de aminoácidos conservados e importantes para a atividade de desaminação de ADO foi detectada (MAIER et al., 2005). Os resultados deste estudo permitem sugerir que, devido à presença de transcritos deste membro em diversos tecidos de zebrafish adulto, esta enzima poderia, de certa forma, estar contribuindo para a clivagem da ADO neste teleósteo.

A presença de dois membros da ADA2 no genoma do zebrafish pode ser remanescente de um evento de duplicação genômica em um determinado período da evolução dos teleósteos (BRUNET et al., 2006). Tanto o gene *ADA2-1* quanto o seu parálogo *ADA2-2* são expressos nos tecidos de zebrafish, sendo que um menor nível relativo de transcrição pode ser atribuído à *ADA2-2*, em virtude da necessidade da utilização da betaína nos experimentos de RT-PCR.

Com relação à *ADA2-1*, foi identificado um splicing alternativo, cuja tradução da seqüência deduzida de aminácidos originou uma proteína truncada (*ADA2-1/T*). O processo conhecido como splicing alternativo é capaz de formar uma ampla variedade de mRNAs, em função dos diferentes arranjos dos exons e dos introns de um determinado gene. Estas distintas combinações podem inclusive originar proteínas truncadas (WANG et al., 2006), as quais podem ter funções biológicas específicas (HAAPASALO et al., 2002; KONNO, 2003). Nossos resultados não permitem afirmar se a *ADA2-1/T* é funcional em zebrafish, porém sua expressão diferencial pode sugerir alguma importância fisiológica ainda não bem estabelecida.

Considerando que a atividade específica da ADA1 de murinos aparentemente possui uma forte correlação com os níveis de mRNA (CHINSKY et al., 1989), a presença de genes relacionados à ADA e as distintas quantidades relativas de transcritos observadas podem ser um indicativo de diferenças funcionais para a ADA1, ADA2-1, ADA2-2 e ADAL nos tecidos de zebrafish.

III.1.3. Caracterização cinética da atividade de desaminação de adenosina em cérebro de zebrafish

Com a finalidade de avaliar o perfil enzimático da clivagem da ADO no SNC de zebrafish, as propriedades bioquímicas da atividade de desaminação deste nucleosídeo foram estudadas em frações solúvel e de membrana. Esta atividade foi linear até 105 min (fração solúvel) e até 180 min (fração de membrana). A curva de proteína demonstrou que a incubação com 1,5 mM de ADO por 75 min e 120 min para as frações solúvel e de membrana, respectivamente, foi linear quando utilizada uma quantidade de proteína na faixa de 5–20 μg.

A influência da temperatura na desaminação da adenosina em cérebro de zebrafish foi avaliada na faixa de 25 a 60ºC (Anexo 2). Os resultados demonstraram que em ambas as frações estudadas a atividade específica aumentou até 45ºC, tendo um declínio significativo na fração solúvel e uma pequena diminuição em membranas a

60ºC. Estes resultados sugerem que a influência da temperatura na atividade de desaminação da adenosina varia de acordo com a localização celular e que as enzimas presentes na fração solúvel são mais sucetíveis a desnaturação. Considerando que a atividade enzimática não é propensa à desnaturação a 37ºC e que estudos prévios realizados no nosso laboratório verificaram que a temperatura sub-ótima para a hidrólise de ATP, ADP e AMP também corresponde a 37ºC (RICO et al., 2003; SENGER et al., 2004), esta foi escolhida para a realização dos ensaios enzimáticos subseqüentes.

O pH ótimo para a desaminação da ADO na fração solúvel foi estimado na faixa de 6,0–7,0, enquanto que a maior atividade enzimática foi encontrada no pH 5,0 em membranas cerebrais. Na literatura, estudos têm demonstrado que a dependência do pH para a reação catalisada pela ADA intestinal em camundongos decresce significativamente em pH abaixo de 5,0 e acima de 9,0 (SINGH & SHARMA, 2000). A comparação das atividades da ADA1 purificada de fígado de humanos e de galinha demonstrou que o pH ótimo de ambas é 7,4 (IWAKI-EGAWA & WATANABE, 2002). Por sua vez, a ecto-ADA de sinaptossomas de cérebro de porcos demonstrou uma ampla faixa de pH ótimo, que variou de 6,0–9,0 (ROMANOWSKA et al., 2007). Estudos relacionados ao outro membro, ADA2, demonstraram que este possui sua atividade específica máxima em pH um pouco mais ácido do que a forma purificada da ADA1 (ZAVIALOV & ENGSTROM, 2005). Embora não se tenham evidências sobre a funcionalidade da ADAL, sua seqüência deduzida de aminoácidos apresenta resíduos altamente conservados e importantes para a atividade enzimática (MAIER et al., 2005; ROSEMBERG et al., 2007b). Desta forma, é provável que a variação de pH encontrada para a desaminação de ADO em cérebro de zebrafish possa ser resultante de uma combinação de enzimas e a preferência pelo pH 5,0 em frações de membrana pode ser fisiologicamente importante em situações de injúrias cerebral, quando os níveis de ADO aumentam e o pH diminui no fluido extracelular (SITKHOVSKY et al., 2004; ZAVIALOV & ENGSTOM, 2005; ELTZSCHIG et al., 2006).

Estudos têm demonstrado que cátions divalentes são capazes de modular a atividade de diferentes membros relacionados à ADA (COOPER et al., 1992; MOHAMED, 2006). Portanto, a influência de metais sobre a atividade de desaminação de adenosina em cérebro de zebrafish foi verificada. Os resultados demonstraram que 5 mM de Zn^{2+} é capaz de promover uma queda significativa na atividade enzimática em frações de membrana. Na literatura, foi demonstrado que o Zn^{2+} atua como um co-fator da ADA, sendo localizado no interior do sítio de ligação do substrato, o qual não é capaz de ser quelado com a adição de EDTA (COOPER et al., 1992; BHAUMIK et al., 1993). Entretanto, foi verificado que o Zn^{+2} quando adicionado ao sistema de incubação pode atuar como um inibidor da atividade enzimática em sítios hidrofílicos mais periféricos (COOPER et al., 1992; MOHAMED, 2006). Nossos dados estão de acordo com estes achados, uma vez que 5 mM de EDTA foi capaz de prevenir o efeito inibitório do Zn^{2+}, o que sugere que este cátion pode interagir em sítios de maior acessibilidade ao solvente da ADA em membranas cerebrais de zebrafish.

Os resultados cinéticos da atividade de desaminação de ADO em cérebro de zebrafish demonstraram que o K_M aparente para as frações solúvel e de membrana é de aproximadamente 0,2 mM, o que está de acordo com resultados previamente publicados para outros organismos (IWAKI-EGAWA &WATANABE, 2002; ROMANOWSKA et al., 2007). Apesar do elevado valor do K_M aparente em relação à concentração fisiológica de ADO (a qual geralmente não excede 30 μM) foi demonstrado que a ADA é necessária para um acoplamento eficiente dos receptores A_1 com proteínas mediadoras da transdução de sinal (CIRUELA et al., 1996). Além disso, estudos têm sugerido que a eficiência da sinalização mediada por A_1 pode ser aumentada com uma diminuição da atividade da ADA (SUN et al., 2005; KEMENY-BEKE et al., 2007). Desta forma, a presença de uma ADA em frações de membrana cerebral de zebrafish também poderia desempenhar um papel extra-enzimático na adesão molecular e também na regulação da sinalização mediada por receptores P1 em condições fisiológicas.

A desaminação de outros substratos, bem como o efeito promovido pelo EHNA foi avaliado neste estudo. Em ambas as frações estudadas, verificou-se uma preferência pela desaminação dos nucleosídeos derivados da adenina (ADO e 2’-D-ADO) em relação aos da guanina (GUO, 2’-D-GUO). Além disso, a presença de 0,1 mM de EHNA induziu uma significativa inibição da atividade de desaminação da adenosina (82,3% e 26,5% em frações solúvel e de membrana, respectivamente). Estudos prévios demonstraram que tanto a ADA1 quanto a ADA2 apresenta uma maior preferência por nucleosídeos de adenina e que o EHNA atua como um inibidor clássico da ADA1 (IWAKI-EGAWA & WATANABE, 2002; IWAKI-EGAWA et al., 2004). Foi sugerido que a ADA1 tipicamente constitui cerca de 90–100% da atividade da ADA intracelular (IWAKI-EGAWA & WATANABE, 2002), o que nos leva a hipotetizar que esta enzima é a principal responsável pela desaminação de ADO na fração solúvel de cérebro de zebrafish. Entretanto, uma vez que não é conhecido o efeito do EHNA na subfamília ADAL, não se pode excluir um efeito inibitório deste composto caso este membro contribua para a desaminação de ADO em zebrafish. A interação de ligantes, tais como substratos e inibidores com a ADAL ainda precisa ser investigada pelo fato da mesma compartilhar similaridades na seqüência deduzida de aminoácidos com os membros clássicos da ADA1 (MAIER et al., 2005; ROSEMBERG et al., 2007b).

III.2. Conclusão final

Com os resultados apresentados nesta Dissertação de Mestrado, nós podemos concluir que o genoma do zebrafish apresenta diferentes membros relacionados à ADA, dos quais incluem a *ADA1*, *ADA2* (*ADA2-1* e *ADA2-2*) e *ADAL*. A análise de expressão gênica demonstrou que estes membros apresentam um padrão de transcrição diferencial entre os tecidos, sendo que uma isoforma truncada de splicing alternativo (*ADA2-1/T*) foi identificada para o gene da *ADA2-1*. Além disso, a caracterização cinética permite sugerir que estes membros podem estar contribuindo para a atividade de desaminação da ADO no cérebro de zebrafish. Tanto na fração solúvel quanto em membranas cerebrais, os resultados demonstraram a existência de uma atividade enzimática capaz de promover a clivagem da ADO, sugerindo que este mecanismo pode ser capaz de promover um ajuste fino na regulação dos níveis deste nucleosídeo.

Portanto, este trabalho contribui para um melhor esclarecimento a respeito dos membros relacionados à ADA em zebrafish. A identificação de diferentes enzimas e a caracterização de uma atividade cinética de desaminação de ADO associada à análise estrutural destes membros poderão ser importantes para a compreensão do sistema adenosinérgico desta espécie.

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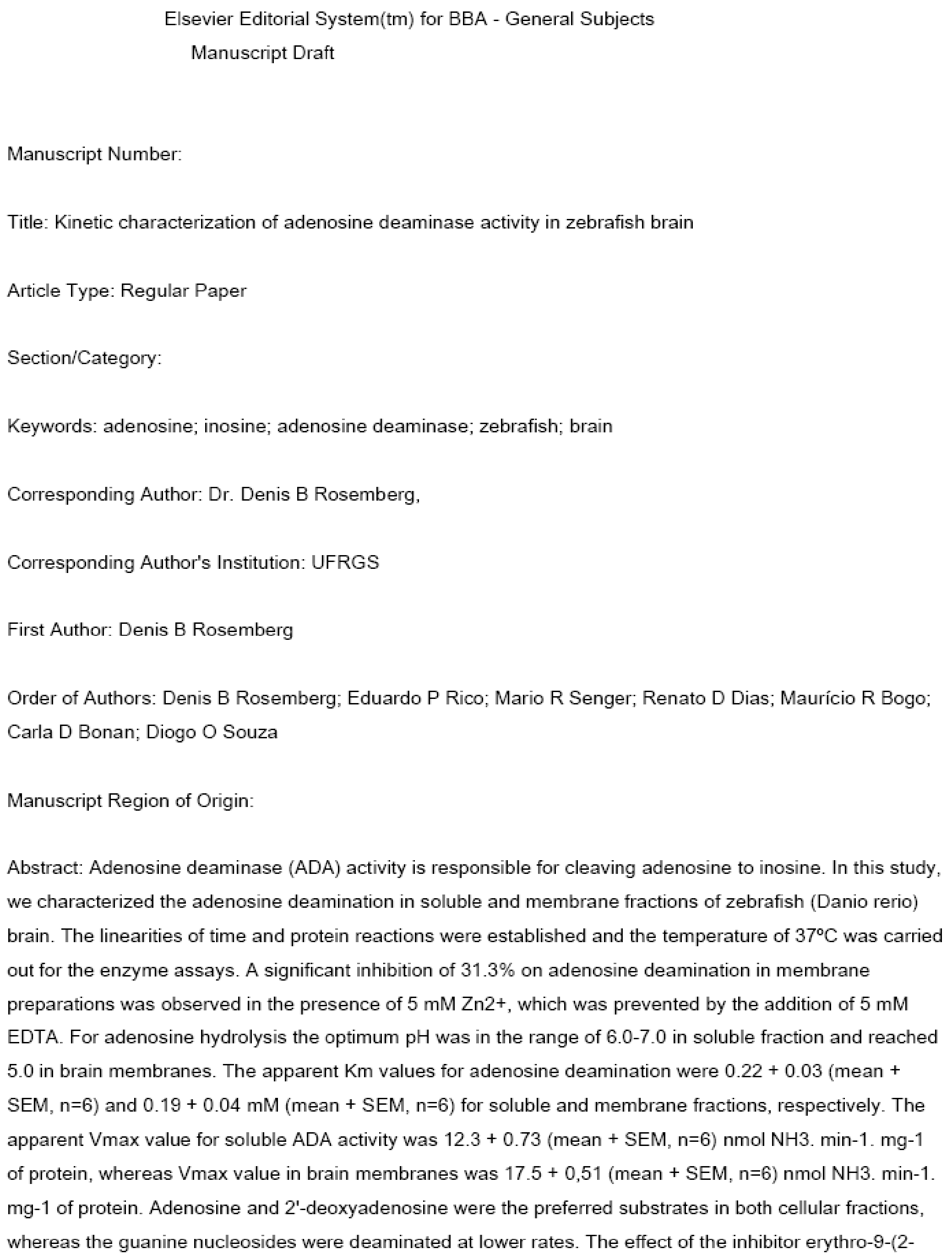
Anexos

Anexo 1: Cópia eletrônica do resumo do manuscrito submetido ao periódico BBA.



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Corresponding Author: Dr. Denis B Rosemberg,

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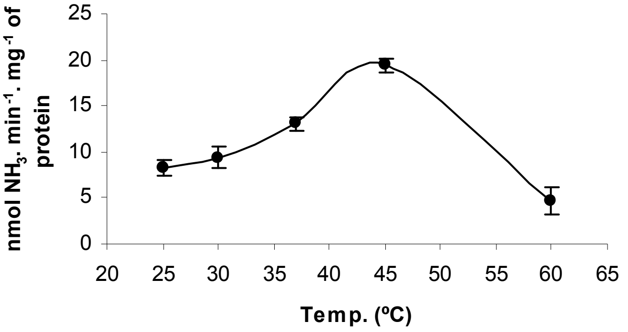
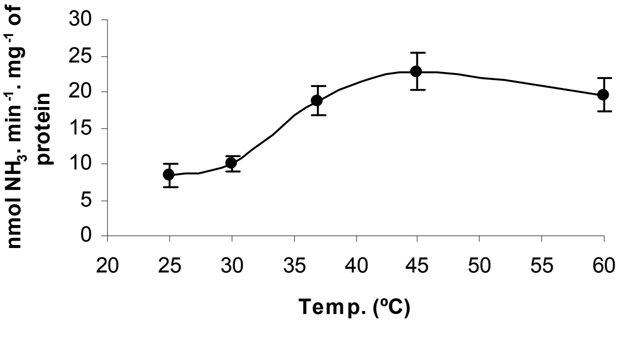
Abstract: Adenosine deaminase (ADA) activity is responsible for cleaving adenosine to inosine. In this study, we characterized the adenosine deamination in soluble and membrane fractions of zebrafish (Danio rerio) brain. The linearities of time and protein reactions were established and the temperature of 37ºC was carried out for the enzyme assays. A significant inhibition of 31.3% on adenosine deamination in membrane preparations was observed in the presence of 5 mM Zn2+, which was prevented by the addition of 5 mM EDTA. For adenosine hydrolysis the optimum pH was in the range of 6.0-7.0 in soluble fraction and reached 5.0 in brain membranes. The apparent Km values for adenosine deamination were 0.22 + 0.03 (mean + SEM, n=6) and 0.19 + 0.04 mM (mean + SEM, n=6) for soluble and membrane fractions, respectively. The apparent Vmax value for soluble ADA activity was 12.3 + 0.73 (mean + SEM, n=6) nmol NH3. min-1. mg-1 of protein, whereas Vmax value in brain membranes was 17.5 + 0,51 (mean + SEM, n=6) nmol NH3. min-1. mg-1 of protein. Adenosine and 2'-deoxyadenosine were the preferred substrates in both cellular fractions, whereas the guanine nucleosides were deaminated at lower rates. The effect of the inhibitor erythro-9-(2-

hydroxy-3-nonyl)adenine (EHNA) was also studied. It was verified that 0.1 mM EHNA promoted a strong decrease on ADA activity in soluble fraction (82.3%) and caused a significant inhibition on adenosine deamination in brain membranes (26.5%). The presence of soluble and membrane-bound ADA activities in zebrafish brain may be important to regulate the ratio of adenosine/inosine nucleosides in both intracellular and extracellular millieu in the CNS of this species.

Anexo 2: Influência da variação de temperatura na atividade de desaminação da adenosina em fração solúvel (A) e de membrana (B) de cérebro de zebrafish.

A



B