The AMP deaminase of the mollusk *Helix pomatia* is an unexpected member of the adenosine deaminase-related growth factor (ADGF) family

George Tzertzinis[1], Mehul B. Ganatra[1], Cristian Ruse[1#], Christopher H. Taron[1], Bryce Causey[1], Liang Wang[1], Ira Schildkraut[1*]

[1] New England Biolabs, Ipswich, MA, 01938, USA

[#]Current Address: Moderna Therapeutics, Cambridge, MA, 02139, USA.

*Corresponding author

E-mail: schildkraut@neb.com

Abstract

We report here the first occurrence of an adenosine deaminase-related growth factor (ADGF) that deaminates adenosine 5' monophosphate (AMP) in preference to adenosine. The ADGFs are a group of secreted deaminases found throughout the animal kingdom that affect the extracellular concentration of adenosine by converting it to inosine. The AMP deaminase studied here was first isolated and biochemically characterized from the roman snail *Helix pomatia* in 1983. Determination of the amino acid sequence of the AMP deaminase enabled sequence comparisons to protein databases and revealed it as a member of the ADGF family. Cloning and expression of its cDNA in *Pichia pastoris* allowed the comparison of the biochemical characteristics of the native and recombinant forms of the enzyme and confirmed they correspond to the previously reported

activity. Uncharacteristically, the *H. pomatia* AMP deaminase was determined to be dissimilar to the AMP deaminase family by sequence comparison while demonstrating similarity to the ADGFs despite having AMP as its preferred substrate rather than adenosine.

Introduction

Adenosine 5' monophosphate (AMP) deaminases catalyze the conversion of AMP to inosine 5' monophosphate (IMP) and ammonia (Fig 1). The AMP deaminases (AMPD) are classified as a subgroup of the adenyl-deaminase family (1) based on their amino acid sequence similarity. The other members of the adenyl-deaminase family belong to the adenosine deaminase (ADA), adenine deaminase (ADE), adenosine deaminase-like (ADAL), and adenosine deaminase-related growth factors (ADGF) subgroups.

The foot muscle of the mollusk, *H. pomatia*, is one of the richest sources (units per mg protein) of AMP deaminase (HPAMPD) (2). HPAMPD was found to convert 5' AMP, 5' ADP, 5' ATP, and NADH to their respective inosine derivatives with low micromolar K_m values (3). These characteristics are unlike those of the animal skeletal muscle AMP deaminases, which have a strict specificity for 5' AMP and relatively high K_m values, >1 mM. Our initial interest in the HPAMPD came from its high activity and its ability to deaminate a di-adenosine tetraphosphate (AppppA) to di-inosine tetraphosphate (IppppI) (4).

For this report we purified HPAMPD and subsequently cloned, sequenced and expressed the cDNA encoding the HPAMPD. We found its amino acid sequence to be most similar to an adenosine deaminase from another mollusk, *Aplysia californica*. The sea slug, *A. californica,* encodes a biologically and biochemically characterized adenosine deaminase which was initially designated as mollusk derived growth factor (MDGF) (5) and later, as a member of the ADGF subfamily (1). The ADGF subfamily is characterized by secreted deaminases which convert the extracellular nucleoside adenosine, a signaling molecule, to inosine. This subfamily of adenyl deaminases is represented in humans as the enzyme encoded by the ADA2 (also known as CECR1) gene. ADA2 is involved in a developmental disorder known as Cat Eye

Syndrome (6). We show, by sequence similarity, that the AMPD from *H. pomatia* is a member of the ADGF subfamily of adenyl deaminases despite the fact that its preferred substrate is AMP. This is the first report of a member of the ADGF subfamily of adenyl-deaminases to prefer the nucleotide AMP over the nucleoside adenosine.

Fig 1. AMP deaminase reaction scheme.

Results

In furthering the characterization of the AMP deaminase of *H. pomatia* we first isolated the native protein from *H. pomatia* foot muscle and determined the sequences of the peptides that comprise it by 2D LC-MS/MS (7,8). Using these peptide sequences, we identified the ORF with the highest peptide sequence coverage from a *H. pomatia* transcriptome. The cDNA which encoded the protein was identified, expressed and the resulting recombinant enzyme characterized.

Purification and characterization of the AMP deaminase from *H. pomatia*

The native HPAMPD was purified from the foot muscle from *H. pomatia* as described in Materials and methods. Deamination was measured by change in absorbance at either OD_{265} or OD_{260} (9). The activity of the HPAMPD for the deamination of the nucleotides AMP, ATP, and adenosine, to their inosine equivalents is shown in Fig 2. The rate of deamination of adenosine and ATP is 0.04 and 0.25, respectively, relative to the rate of AMP. The specific activity of the native HPAMPD preparation was 125 units per mg of protein. However, the protein preparation appeared by PAGE

71 analysis (S1 Fig.) to comprise a protein of 60 kD and contaminating proteins of 32 and 27 kD. This
72 result is consistent with the monomer size reported by Stankiewicz (2) at 60-64 kD with an
73 additional two proteolytic degradation products of the monomer observed here. The specific
74 activity reported by Stankiewicz (10) was similar at 117 units per mg protein.
75
76 **Fig 2. Relative rates of deamination.** Activity of native *H. pomatia* AMP deaminase on 100 μM 5'
77 AMP, 5' ATP, and adenosine. The reaction volume was 0.4 ml. The change in absorbance at OD_{265}
78 was followed in a spectrophotometer at 25° C.
79

80 Peptide analysis by mass spectrometry

81 The purified HPAMPD protein was analyzed using a two-dimensional LC MS/MS system (see
82 Materials and methods). The *de novo* peptides derived from the mass spectrometry were
83 intersected with open reading frames (ORFs) that were obtained by translating the *H. pomatia*
84 transcriptome. Peptide scoring validation was based on $q<0.01$ (11). Using the peptide sequences,
85 we identified the ORF from the transcriptome with the highest peptide sequence coverage. The
86 peptides covered 67% of a 536 amino acid ORF encoded by contig 71391 (Fig 3). The list of
87 identified peptides from analyses of the digests is detailed in Supporting Information (Table S1).
88
89 **Fig 3. The amino acid sequence of the *H. pomatia* transcriptome contig 71391**. The sequence
90 segments covered by LC-MS/MS derived peptides are shown in blue text.

91 In order to determine the sequence and obtain a clone for expressing the protein in a recombinant
92 form we generated cDNA from *H. pomatia* RNA. The cDNA was used as a template for PCR
93 amplification using primers designed based on the sequence of contig 71391. The resulting 1596 bp
94 fragment was sequenced and inserted into the pET28c vector (see Material and methods). Several

nucleotide positions in the cDNA differed from the transcriptome which resulted in nine amino acid differences from contig 71391 presumably because the transcriptome had been derived from a different population of *H. pomatia* (S2 Fig.).

The contig 71391 encodes for a protein with an amino terminal end consistent with a signal peptide as predicted by SignalP-4.1 (12) of MSQVGAVTMVSIICVVVLGAVGGPVAG which *in vivo* should result in a secreted protein with either an amino terminal sequence of AVGGPVAGLAVRFPT or LAVRFPT. Both alternative amino terminal versions of the processed protein were submitted to structure prediction by ColabFold (13). The resulting structures indicated that in the protein with the additional 8 terminal residues, AVGGPVAG, did not appear to be a structural feature of consequence (S3 Fig.).

HPAMPD is a member of the ADGF subfamily of adenyl-deaminases.

The generation of a phylogenetic tree based on amino acid sequence reveals HPAMPD to cluster within the ADGF subfamily of the adenyl-deaminase family (Fig 4). Consistent with the ADGF subfamily, the signal peptide of HPAMPD is a characteristic indicating the protein is secreted. InterPro (14) analysis of the HPAMPD amino acid sequence also indicated it is a member of the adenosine deaminase-related growth factors (ADGF). The determination that the HPAMPD was a member of the ADGF subfamily of adenosine deaminases rather than a member of the AMPD subfamily was surprising because of its preference for AMP over that of adenosine as substrate.

116 **Fig 4. Phylogenetic tree of the adenyl-deaminase family of proteins.** The tree was generated
117 with FastTree (15) from a MAFFT alignment. The numerical values at each branch represent
118 substitutions per site. ADGF are the adenosine deaminase-related growth factors, AMPD are the
119 AMP deaminases, ADE are the adenine deaminases, ADAL are the adenosine deaminase-like
120 proteins, ADAR are the adenosine deaminases acting on RNA, and the ADA are the adenosine
121 deaminases. The substrate assigned to each subfamily is indicated on the right. The accession
122 numbers for each enzyme follow: ADGF human, AAF65941.1; ADGF Aplysia, AAD13112.1; ADGF
123 Fish, AF384217.1; ADGF Lottia, XP_009053965.1; ADGF Chicken, AAX10953.1; ADGF Frog,
124 AAX10952.1; ADAL Arabidopsis, NP_192397.2; ADAL human iso3, NP_001311295.1; ADE
125 Aspergillus, AL56636.1; ADE Saccharomyces, NP_014258.1; ADA C elegans, NP_872091.1; ADA
126 human iso1, NP_000013.2; AMPD human, NP_000471.1; AMPD Arabidopsis, NP_565886.1; AMPD2
127 human, NP_001244290.1; AMPD Drosophila, NP_727740.2.

128

129 Observing that HPAMPD is a member of the ADGF subfamily, we used MAFFT (16) to align HPAMPD
130 with representatives of the ADGF subfamily (Fig 5) which includes the most similar member, *A.*
131 *californica* ADGF, with an identity score of 57% and an additional mollusk, *Lottia gigantea*. The
132 other members span the vertebrates. Fortuitously, there is an experimentally derived three-
133 dimensional structure for the human ADGF, with an identity score of 38%. This allowed us to
134 superimpose it with the predicted HPAMPD structure.

135

136 **Fig 5. Alignment of the HPAMPD to other ADGF subfamily members.** Underlined residues are
137 predicted signal sequences (17) and blue outlined boxes are catalytic residue positions defined by
138 (18) in human ADA2. HPAMPD sequence is derived from cDNA.

139

140 # Structure comparison of HPAMPD to human and *A. californica*
141 # ADGF

142 Structures of the HPAMPD and *A. californica* ADGF were predicted with AlphaFold2 (13,19) and
143 were compared to the reported experimentally derived human ADGF structure, 3LGG, (18)
144 obtained from the Protein Data Bank (PDB) (20). The PDB files were visualized and aligned with
145 Pymol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.)(Fig 6a). HPAMPD is
146 aligned to *A. californica* ADGF with a RMSD of 0.594 and to human ADGF with an RMSD of 0.777.
147 Structure alignments of the PDB files with Pymol demonstrate the close structural relatedness of
148 HPAMPD to the two ADGFs. The active site of the human ADGF is elucidated because it formed a
149 cocrystal with coformycin, a transition state analog and potent inhibitor of adenosine deaminases.
150 Based on the structure of the human ADGF, which harbors a tightly coordinated zinc ion at the base
151 of its active site (18) it is expected that the HPAMPD also contains a zinc ion as all four of the zinc-
152 coordinating residues of the human sequence are conserved and aligned in the HPAMPD structure
153 (Fig 6b).

154

155 **Fig 6. Structural Comparison of HPAMPD and human ADGF.** (A) A superimposition of the
156 AlphaFold2 predicted HPAMPD monomer and a monomer of the human ADGF (PDB 3LGG). Human
157 ADGF is colored pink and the HPAMPD is colored cream. Side chains of active site residues and
158 coformycin (colored blue) are drawn as sticks. The zinc ion is represented as a sphere and dashed
159 lines depict interactions with coordinating residues from human ADGF. (B) A close-up view of the
160 active site residues, zinc and coformycin. The labeled residues correspond to the 3LGG structure.

161

162 ## Cloning the cDNA encoding HPAMPD for expression in *Pichia*
163 ## *pastoris*

164 Because the ADGFs as a group are extracellular eukaryotic secreted proteins, we utilized the
165 budding yeast *Pichia pastoris* expression system (21) to express and secrete HPAMPD (see
166 Materials and methods). The processed amino terminus of the HPAMPD is engineered to start at
167 residues LAVR by use of the N-terminal secretion signal from *S. cerevisiae* alpha-factor. The carboxy
168 end is terminated with 10 amino acid histidine residues. The amino acid sequence of the protein as
169 secreted from *P. pastoris* is shown in Fig 7. The molecular weight of the protein as expressed in *P.*
170 *pastoris* is 58676 daltons which is consistent with the value from SDS-PAGE of 60 kD (S1 Fig.). The
171 specific activity of the recombinant HPAMPD preparation was determined to be 550 units/mg of
172 protein.
173
174 **Fig 7. The amino acid sequence of the HPAMPD cDNA clone expressed in *P. pastoris*.**
175

176 ## Characterization of recombinant HPAMPD activity

177 The recombinant form of the HPAMPD has a similar spectrum of activity on adenyl substrates as
178 the native enzyme preparations from this study and (2) (Table 1). All three 5' nucleotides, AMP,
179 ADP and AMP are actively deaminated while the absence of a phosphate at the 5' position lowers
180 the activity substantially. Adenosine, 3'5'-cyclic AMP and 3' AMP are poor substrates. This profile is
181 consistent with the presumption that the native and recombinant forms of HPAMPD are one and
182 the same enzyme and represent the same enzyme characterized in 1983.
183
184

Substrate	Relative activity from (Stankiewicz 1984) 70 μM substrate	Relative activity of native 100 μM substrate	Relative activity of recombinant 100 μM substrate
5' AMP	100	100	100
5' ADP	95	83	74
5' ATP	36	25	ND
3'5'-cyclic AMP	0.35	2	0.5
adenosine	10 (@3 mM adenosine)	4	2.8
3'-5' adenosine diphosphate (PAP)	8 (@ 3mM PAP)	1.6	0.7
3' AMP	ND	0.9	< 0.1

185 **Table 1. Relative rates of deamination.** Spectrophotometric assay of deamination of adenyl base
186 monitored by loss of OD_{260} or OD_{265}. The rates are linear initial rates and expressed as a percentage
187 of the 5' AMP rate. Reactions were at 25°C in 20 mM Tris-HCl pH 7.5, 50 mM NaCl and the indicated
188 concentration of each substrate.

189

190 Kinetic constants for recombinant HPAMPD and AMP, K_m and K_{cat}, were determined from linear
191 initial rates of reaction where 57.5 fmoles of enzyme were incubated in 200 μL reactions with
192 various concentrations of AMP. The K_m of 25 μM and K_{cat} (turnover number) of 170 per second are
193 derived from the Lineweaver-Burk plot (Fig 8). Stankiewicz (3) reported a K_m value of 31 μM for 5'
194 AMP.

195 **Fig 8. Lineweaver-Burke plot for determination of V_{max} and K_m values for recombinant**
196 **HPAMPD with AMP.**
197
198 Preincubation of the enzyme at 65° C in the absence of substrate in 10 mM Tris-HCl pH 7.5, 50 mM
199 NaCl, 1 mM EDTA for 30 minutes reduces its activity by more than 95%, while 42° C preincubation
200 has no effect on activity (S4 Fig.). Analysis of thermal denaturation with Prometheus (Nanotemper)
201 indicates the HPAMPD initiates denaturation at 47° C and melts (50 % unfolded) at 54 ° C.

202

203 Comparison of *A. californica* and human ADGF activities on
204 adenosine and AMP

205 The determination that the HPAMPD was a member of the ADGF subfamily of adenosine
206 deaminases was surprising because of its preference for AMP over that of adenosine as substrate.
207 This led us to question the annotation of ADGFs, as we could find only one biochemical evaluation
208 of the deamination of AMP (less than 1%) relative to adenosine in the literature (22). Therefore, we
209 directly compared the activities of HPAMPD, A. *californica* and human homologues on both
210 adenosine and AMP. The relative initial rates of the reactions are listed in Table 2. The results
211 substantiate the assertion that the human and *A. californica* ADGFs deaminate adenosine and do not
212 deaminate AMP, whereas HPAMPD deaminates AMP and does not significantly deaminate
213 adenosine.

	AMP	Adenosine
HPAMPD	1 (1)	0.03 (0.04)

Aplysia ADGF	0.001	1
Human ADGF	< 0.01	1

214 **Table 2. Relative initial rates of deamination of adenosine and AMP for each deaminase.** The
215 deamination was measured by loss of OD_{265} in 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 50 mM NaCl
216 buffer. The substrate concentrations were 100 μM for the HPAMPD and *A. californica* enzymes and
217 2 mM for the human enzyme. The reaction temperature for *H. pomatia* and *A. californica* ADGFs was
218 25° and 37° for human ADGF. All values were obtained with recombinant enzymes except for the
219 value in parentheses which was obtained with the native preparation of HPAMPD.

220

221 # Discussion

222 Here, we report for the first time the identification of an ADGF family member whose substrate
223 preference is adenosine 5' monophosphate. The biochemical characterization indicates that
224 HPAMPD deaminates AMP with a K_m of 25 μM and a K_{cat} of 170 per second indicating that this is one
225 of the more efficient reactions among the adenosine and AMP deaminases. We present evidence
226 that the AMP deaminase reported in 1983 (3) is the same as the one cloned here from *H. pomatia*.
227 HPAMPD is unlikely to be the only ADGF which prefers AMP. The few ADGFs which have been
228 biochemically examined have been shown to deaminate adenosine. Here we demonstrate that the
229 ADGFs most closely related to HPAMPD, the *A. californica* ADGF and the human ADGF prefer
230 adenosine to AMP as substrate. ADGFs listed in protein databases are annotated as such primarily
231 on the basis of sequence similarity. Prediction of gene annotations ideally should utilize full
232 descriptions of the encoded proteins' biochemical properties, that is in the case of an enzyme, its
233 substrates and those related compounds that are not (23). However, in practice, that is

234 understandably an extremely difficult requirement to fulfill. Among the 1777 ADGF family
235 members listed in the UniProtKB database (Uniprot consortium) only 7 members have been
236 manually annotated by Swiss-Prot. Of the manually annotated members only 4 have been assayed
237 for activity on adenosine and only one of those on AMP for which there was less than 1% of the
238 activity as there was on adenosine (22). Based on the results of our study, it is probable that some
239 fraction of the 1777 ADGF members prefer AMP as substrate and are therefore incorrectly
240 annotated.
241 We suggest that the inability of the human and Aplysia ADGFs to deaminate AMP is not due to the
242 positioning of the catalytic residues but rather may reflect a steric clash and/or charge repulsion of
243 the AMP phosphate. The HPAMPD has the same positioning of the catalytic residues as human
244 ADGF. Clearly the same chemistry is taking place for deamination for all the ADGFs. The HPAMPD
245 preference for AMP could be explained with a binding pocket that can accommodate the phosphate
246 of AMP which contributes to substrate binding. Further work in this lab will attempt to elucidate
247 the basis for the adenosine vs. AMP preference.
248
249 The ADGFs are involved in purinergic signaling. They are characterized as secreted growth factors
250 and affect growth by modulating the extracellular levels of adenosine. We have shown that
251 HPAMPD has an encoded signal peptide. Therefore, it is likely secreted in *H. pomatia,* and its site of
252 action is extracellular. We propose that HPAMPD affects the level of extracellular AMP and is
253 involved in purinergic signaling in some as yet unknown role. Skaldin (24) reported a bacterial
254 ADGF homologue which is secreted and deaminates adenosine and suggested that the ADGFs
255 evolved from a secreted bacterial homologue with a similar role involved in bacterial
256 communication.
257

258 The occurrence of purinoreceptors in primitive invertebrates suggests that ATP is an early
259 evolutionary extracellular signaling molecule (25,26). Perhaps *H. pomatia* uses an alternative
260 pathway for modulating extracellular levels of ATP. For example, rather than converting ATP to
261 AMP and then AMP to adenosine and deaminating adenosine to inosine as a last step, *H. pomatia*,
262 removes AMP directly by deamination to IMP by HPAMPD as a last step.
263
264 In conclusion, HPAMPD is an AMP deaminase that is assigned by sequence similarity to the
265 adenosine deaminase-related growth factor family which suggests that there is a biological role of
266 AMP deaminases as growth factors. Furthermore, HPAMPD is a prime example for illustrating that
267 the assignment of an enzymatic activity based only on sequence similarity in absence of
268 biochemical evidence is imprudent.
269

270 Materials and methods

271 Purification of native AMPD from *H. pomatia*

272 The *H. pomatia* were obtained from My Happy Snails (myhappysnails.com) and fed on romaine
273 lettuce for 5 days. The foot muscle from 2 snails was dissected. 1 gram was stored at -70° C and was
274 later used for RNA extraction and cDNA cloning. The remaining 5 grams was homogenized in 20 ml
275 of 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 1M NaCl. The homogenate was clarified by
276 centrifugation and NaCl concentration was reduced to 200 mM by dilution with 20 mM Tris-HCl pH
277 7.5, 1 mM EDTA, 1 mM DTT. The clarified extract contained 600 AMP deaminase units.
278 The clarified extract was applied to a 5 ml Q sepharose column where 80% of the AMP deaminase
279 flowed through the column. The Q flow-through was applied to a 5 ml HiTrap™ Heparin column. A
280 gradient of NaCl was run to 1.2 M and the AMPD activity eluted at about 0.4M NaCl. The peak of

281 activity was pooled and diluted to 0.1 M NaCl and subsequently applied to a 5 ml HiTrap™ Q
282 column in 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1 M NaCl. The activity flowed through
283 the column and was then applied directly onto a 5 ml HiTrap™ SP column. A gradient to 1 M NaCl
284 was run and the activity eluted at about 0.5 M NaCl. An aliquot (10 μL) of a peak fraction was
285 subjected to *de novo* peptide sequence analysis. The remainder of the AMP deaminase active
286 fractions were pooled and represent the native enzyme preparation with a yield of 30% at 200
287 units with a specific activity of about 160 units/mg protein.

288

289 AMPD assay

290 The assay is based on Kalckar's spectrophotometric method (9) where the decrease in optical
291 density at 265 nM is measured. The reaction buffer is 20 mM Tris-HCl @ pH 7.5, 50 mM NaCl and
292 typically the substrate concentration is 100 μM AMP. The reaction volume ranged from 200 to 400
293 μL. The optical density was determined with a Spectramax M3. One unit of the enzyme activity is
294 defined as the amount of the enzyme which deaminates 1 μmole of 100 μmolar 5' AMP/min in 20
295 mM Tris-HCl pH7.5, 50 mM NaCl at 25° C.

296

297 ## *H. pomatia* transcriptome

298 The *H. pomatia* snails were obtained from a farmers' market in Germany and stored at -80°C.
299 Approximately 100 mg of *H. pomatia* body were frozen in liquid nitrogen and ground to a powder.
300 Total RNA was purified from rehydrated powder with the RNeasy Plant Mini Kit (Qiagen,
301 Chatworth, CA). An mRNA transcript library for Illumina sequencing was created using the
302 NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (New England Biolabs, Ipswich, MA). A NextSeq
303 500 sequencer (Illumina, San Diego, CA) was used to do paired-end deep sequencing (2X 150 bp) of
304 the library. The utility CutAdapt (27) was used to remove adapter sequences from the raw reads.
305 The trimmed reads were then assembled using the Trinity software package (28). The assembled
306 transcriptome data was deposited at DDBJ/EMBL/GenBank under the accession GKIM00000000.
307 The version described in this paper is the first version, GKIM01000000. Raw reads were deposited
308 in the NCBI Sequence Read Archive (accession #PRJNA936131).

309

310 ## Peptide sequencing by 2D LC-MS/MS

311 The purified HPAMPD protein fraction was digested in solution in parallel, with either trypsin at a
312 ratio of trypsin to protein at 1:50 or subtilisin at a ratio of 1:160. The subtilisin digested fraction
313 was analyzed twice, to include charge state 1 peptides. The trypsin digest was analyzed with one
314 injection. Each digest was directly injected onto an analytical C18 column and analyzed with Easy
315 n1000 nLC-LTQ Orbitrap (Thermo Fisher Scientific, Waltham, MA) at 300 nL/min. All MS/MS
316 spectra were analyzed with ProteomeDiscoverer version: 2.0.0.802 in one workflow. The fasta
317 database consisted of open reading frames translated from the *H. pomatia* transcriptome. Peptide
318 scoring validation was based on $q<0.01$ (11).

319

320 ## RNA isolation for cDNA

321 The source of *H. pomatia* for cDNA was the same as for the HPAMPD enzyme preparation. About 1
322 gram of frozen *H. pomatia* foot tissue was pulverized in liquid nitrogen with mortar and pestle.
323 About 0.2 grams of powdered tissue was homogenized in 1 ml of TRIzol reagent (Thermo Fisher).
324 The debris was pelleted, and 0.2 ml of chloroform was added per ml of supernatant vortexed for 30
325 sec and phases separated by a 15 min centrifugation. 1 volume of ethanol was added to the aqueous
326 layer and the mixture was applied to and eluted from a Direct-zol column (Zymo RNA miniprep kit)
327 yielding 25 μg of RNA.

328

329 ## cDNA cloning

330 First strand cDNA was generated from *H. pomatia* RNA with Protoscript II (NEB) and random
331 primers. The first strand cDNA was used as a template for PCR amplification using primers A and B
332 (see below) which were designed based on the transcriptome derived sequence for HPAMPD. The
333 resulting 1556 bp fragment was inserted into the pET28c vector digested with EcoRI and NcoI
334 using the NEBuilder HiFi Assembly kit (NEB).

335 Primer sequences:
336 A: ACT TTA AGA AGG AGA TAT ACC ATG CTC GCC GTC AGA TTT CCG
337 B: AAG CTT GTC GAC GGA GCT CGT ACC GAT AAC TTT CTT GTT GGT TAG

338

339 # Construction of expression cassette for *P. pastoris*

340 pD912-P_{GAP} plasmid was obtained from ATUM Bio (Newark, CA). A linear expression cassette of
341 pD912-P_{GAP}-Hp AMPD-His_{10} was assembled using the NEBuilder HiFi DNA Assembly Cloning Kit
342 (NEB #E5520S). Primers were designed using the NEBuilder Assembly Tool. The HPAMPD
343 fragment was amplified with the forward primer C and reverse primer D (containing a 10X His-tag
344 sequence) using plasmid pET28c-Hp AMPD as template. The pD912-P_{GAP} fragment containing the
345 transcriptional terminator (TT), ILV5 promoter, and zeocin resistant gene was amplified with the
346 forward primer E and reverse primer F using pD912-P_{GAP} plasmid as template. All products were
347 purified by gel extraction. The linear expression cassette was then amplified with forward primer G
348 and reverse primer H. The gel extracted product was used to transform electrocompetent *P.*
349 *pastoris* D aox1 (MutS) cells (ATUM Bio.) and plated on yeast peptone dextrose agar medium
350 supplemented with 1 M sorbitol and 500 μg/mL Zeocin (Teknova, Hollister, CA.).
351 Eight to twelve transformants were patched onto fresh selection plates and incubated for an
352 additional 1-2 days at 30°C. For the identification of transformants by PCR, genomic DNA was
353 isolated from each strain using lithium acetate/sodium dodecyl sulfate (LiOAc/SDS) method (29).
354 PCR was used to identify transformants having an integrated expression cassette.
355 Primer sequences:
356 C: CGA GAA AAG AGA GGC CGA AGC TCT CGC CGT CAG ATT TCC G
357 D: TTG AGC GGC CGC CCC TTC AAC CTC AGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GAC CGA
358 TAA TTT CTT GTT GGT TAG
359 E: CAC CAC CAC CAC CAC CAC CAC CAC CAC CAC TGA GGT TGA AGG GGC GGC CGC
360 F: AGC TTC GGC CTC TCT TTT C
361 G: GCT CAT TCC AAT TCC TTC TAT TAG
362 H: GAG CTC CAA TCA AGC CCA ATA AC
363

364 ## *P. pastoris* clone harboring HPAMPD cDNA

365 The *P. pastoris* transformants that had integrated the expression cassette coding for the HPAMPD
366 were grown in liquid cultures at 30°C in 5 mL of BMGY-Buffered Glycerol Complex Medium
367 (Teknova) (1% yeast extract, 2% tryptone, 1.34% yeast nitrogen base without amino acids with
368 ammonium sulfate, 0.0004% biotin, 1% glycerol as the carbon source, 100 mM potassium
369 phosphate, pH 6.0). After 48 hours, the spent culture media was harvested. The culture fluid from 4
370 cultures one of which was a non-transformant control culture were each concentrated about 10-
371 fold and assayed for AMP deaminase activity. One transformant had no detectable deaminase
372 activity while the other two converted about 25% and 90% of the AMP to IMP in 4 minutes at 25° C.
373

374 ## Purification of recombinant HPAMPD

375 The culture identified with the highest HPAMPD activity was then grown in 2400 ml of Tolner
376 media (30) with 1% glycerol and 100 μg/ml ampicillin at 30° C for 72 hours. The culture was
377 clarified by centrifugation and the supernatant culture fluid was brought 75% ammonium sulfate
378 saturation and centrifuged to clarify. The purification was performed at 4° - 10° C except
379 where noted. The 3.5-liter supernatant was then diluted with 700 ml of water and applied to a 250
380 ml bed Phenyl Sepharose column. After loading the column with supernatant, the column was
381 washed with 500 ml of 3M ammonium sulfate, 20 mM Tris-HCl pH 7.5, 1mM EDTA and 1 mM DTT.
382 A reverse gradient of 1800 ml from 2M to 0 M ammonium sulfate was applied to the column. The
383 HPAMPD activity that eluted from the column was pooled (120 ml) and diluted with 2 liters of 20
384 mM Tris-HCl pH 7.5, 25 mM NaCl, 1mM EDTA, 1 mM DTT. This was applied to a 10 ml Heparin
385 HyperD column washed with 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 50 mM NaCl and
386 eluted with a 400 ml gradient from 50 mM to 1 M NaCl in 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1
387 mM DTT. The peak of activity was pooled and half of the pool was treated with Endo Hf (NEB) by

388 first dialyzing the 25 ml against 2 changes of 500 ml of 20 mM Na acetate pH 6.0, 1 mM EDTA, 50
389 mM NaCl, 1 mM DTT. Five hundred thousand units of EndoHf was added to the 25 ml dialysate and
390 incubated at 25° C overnight. A small amount of precipitate formed and was pelleted and removed
391 by centrifugation. In order to remove the EndoHf from the HPAMPD the supernatant was brought
392 to 100 mM Tris -HCl pH 7.5 and 100 mM NaCl and applied to a one ml amylose resin (NEB) column
393 at a rate of about 1 drop per second at 25° C. The flow through containing the HPAMPD was then
394 applied directly to a 5 ml HiTrap™ Q column in line with a 5 ml HiTrap™ SP column and washed
395 with 20 mM Tris-HCl pH 7.5, 1mM EDTA and 1 mM DTT, 50 mM NaCl. The Q column was removed
396 and a 50 ml gradient from 50 mM to 1M NaCl was applied to the SP column. The peak of HPAMPD
397 activity was pooled and dialyzed against 50% glycerol, 10 mM Tris-HCl pH 7.4, 1 mM DTT, 0.1 mM
398 EDTA. The recombinant HPAMPD preparation is stored at -20° C. The protein is >95%
399 homogenous by PAGE (S1 Fig.) and protein concentration is 2.6 mg/ml based on OD_{280} with
400 an extinction coefficient of 85,000 M^{-1} cm^{-1}.
401

402 Human and *A. californica* ADGF

403 The human ADGF (Recombinant Human Adenosine deaminase 2/CECR1) #7518-AD-010 was from
404 R&D Systems, Minneapolis, MN and the *A. californica* ADGF was purified from construct described
405 below. The *P. pastoris* clone carrying the *A. californica* ADGF cDNA was grown and purified as
406 described for the *H. pomatia* construct except deglycosylation was not performed.
407

408 Yeast culture conditions and expression

409 Construction of the *A. californica* construct for expression in *P. pastoris* was performed as follows:
410 The transformation cassette for *A. californica* ADGF, was generated by PCR using primers 1941 and

411 1942 and as a template the clone in PD912-GAP which was obtained from Genscript, Piscataway, NJ.
412 The PCR product was confirmed for size by agarose gel electrophoresis and subsequently purified
413 by QIAquick (Qiagen) columns. The purified product (1 μg) was electroporated into *P. pastoris*
414 competent cells and positive colonies identified as described above.
415 Primer sequences:
416 1941: GCT CAT TCC AAT TCC TTC TAT TAG
417 1942: GAG CTC CAA TCA AGC CCA ATA AC
418

419 # Acknowledgments

420 We thank Sean Johnson for a critical reading of the manuscript, the DNA sequencing core lab (NEB),
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423

424 # References

425 1. Maier SA, Galellis JR, McDermid HE. Phylogenetic Analysis Reveals a Novel Protein Family
426 Closely Related to Adenosine Deaminase. J Mol Evol. 2005 Dec;61(6):776–94.

427 2. Stankiewicz AJ. Non-specific Adenylate Deaminase from Snail Foot Muscle. Adva Exp Med Biol.
428 1984;165 pt B:457–64.

429 3. Stankiewicz AJ. Direct deamination of AMP, ADP, ATP and NADH by non-specific adenylate
430 deaminase in the foot muscle of the snail *Helix pomatia*. Biochem J. 1983 Oct 1;215(1):39–44.

431 4. Guranowski A, Starzynska, E, Sillero, MA Gunther, Sillero, A. Conversion of adenosine 5'
432 oligophospho 5' adenosines into inosine 5" oligophospho 5' inosines by non-specific adenylate
433 deaminase from the snail Helix pomatia. Biochim Biophys Acta. 1995;1234:78–84.

434 5. Akalal DBG, Nagle GT. Mollusk-derived growth factor: cloning and developmental expression in
435 the central nervous system and reproductive tract of Aplysia. Mol Brain Res. 2001 Jul;91(1–
436 2):163–8.

437 6. Riazi MA, Brinkman-Mills P, Nguyen T, Pan H, Phan S, Ying F, et al. The Human Homolog of
438 Insect-Derived Growth Factor, CECR1, Is a Candidate Gene for Features of Cat Eye Syndrome.
439 Genomics. 2000 Mar;64(3):277–85.

440 7. Bouzid W, Verdenaud M, Klopp C, Ducancel F, Noirot C, Vétillard A. De Novo sequencing and
441 transcriptome analysis for Tetramorium bicarinatum: a comprehensive venom gland
442 transcriptome analysis from an ant species. BMC Genomics. 2014;15:987.

443 8. Evans VC, Barker G, Heesom KJ, Fan J, Bessant C, Matthews DA. De novo derivation of
444 proteomes from transcriptomes for transcript and protein identification. Nat Methods. 2012
445 Dec;9(12):1207–11.

446 9. Kalckar HM. Differential spectrometry of purine compounds by means of specific enzymes. J
447 Biol Chem. 1947 Feb;167(2):461–75.

448 10. Stankiewicz A. Nonspecific Snail Muscle Adenylate Deaminase: Simplified Purification,
449 Characterization and Use for the Preparation of Deamino Derivatives of NAD, NADH and AMP-
450 P(NH)P. Enzyme. 1986;36(3):187–96.

451 11. Savitski MM, Wilhelm M, Hahne H, Kuster B, Bantscheff M. A Scalable Approach for Protein
452 False Discovery Rate Estimation in Large Proteomic Data Sets. Mol Cell Proteomics. 2015
453 Sep;14(9):2394–404.

454 12. Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from
455 transmembrane regions. Nat Methods. 2011 Sep 29;8(10):785–6.

456 13. Mirdita M, Schütze K, Moriwaki Y, Heo L, Ovchinnikov S, Steinegger M. ColabFold: making
457 protein folding accessible to all. Nat Methods. 2022 Jun;19(6):679–82.

458 14. Paysan-Lafosse T, Blum M, Chuguransky S, Grego T, Pinto BL, Salazar GA, et al. InterPro in 2022.
459 Nucleic Acids Res. 2023 Jan 6;51(D1):D418–27.

460 15. Price MN, Dehal PS, Arkin AP. FastTree 2 – Approximately Maximum-Likelihood Trees for Large
461 Alignments. Poon AFY, editor. PLoS ONE. 2010 Mar 10;5(3):e9490.

462 16. Katoh K, Standley DM. MAFFT Multiple Sequence Alignment Software Version 7: Improvements
463 in Performance and Usability. Mol Biol Evol. 2013 Apr 1;30(4):772–80.

464 17. Teufel F, Almagro Armenteros JJ, Johansen AR, Gíslason MH, Pihl SI, Tsirigos KD, et al. SignalP
465 6.0 predicts all five types of signal peptides using protein language models. Nat Biotechnol.
466 2022 Jul;40(7):1023–5.

467 18. Zavialov AV, Yu X, Spillmann D, Lauvau G, Zavialov AV. Structural Basis for the Growth Factor
468 Activity of Human Adenosine Deaminase ADA2. J Biol Chem. 2010 Apr 16;285(16):12367–77.

469 19. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, et al. Highly accurate protein
470 structure prediction with AlphaFold. Nature. 2021 Aug 26;596(7873):583–9.

471 20. Berman H, Henrick K, Nakamura H. Announcing the worldwide Protein Data Bank. Nat Struct
472 Mol Biol. 2003 Dec;10(12):980–980.

473 21. Sreekrishna K, Potenz RHB, Cruze JA, McCombie WR, Parker KA, Nelles L, et al. High level
474 expression of heterologous proteins in methylotrophic yeast Pichia pastoris. J Basic Microbiol.
475 1988;28(4):265–78.

476 22. Iijima R, Kunieda T, Yamaguchi S, Kamigaki H, Fujii-Taira I, Sekimizu K, et al. The Extracellular
477 Adenosine Deaminase Growth Factor, ADGF/CECR1, Plays a Role in Xenopus Embryogenesis
478 via the Adenosine/P1 Receptor. J Biol Chem. 2008 Jan;283(4):2255–64.

479 23. Zhao Y, Wang J, Chen J, Zhang X, Guo M, Yu G. A Literature Review of Gene Function Prediction
480 by Modeling Gene Ontology. Front Genet. 2020 Apr 24;11:400.

481 24. Skaldin M, Tuittila M, Zavialov AV, Zavialov AV. Secreted bacterial adenosine deaminase is an
482 evolutionary precursor of adenosine deaminase growth factor. Perna N, editor. Mol Biol Evol.
483 2018 Oct 16;35(12):2851–61.

484 25. Vultaggio-Poma V, Falzoni S, Salvi G, Giuliani AL, Di Virgilio F. Signalling by extracellular
485 nucleotides in health and disease. Biochim Biophys Acta BBA - Mol Cell Res. 2022
486 May;1869(5):119237.

487 26. Burnstock G. Purine and purinergic receptors. Brain Neurosci Adv. 2018
488 Jan;2:239821281881749.

489 27. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads.
490 EMBnet.journal. 2011;17:10–2.

491 28. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length
492 transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 2011
493 Jul;29(7):644–52.

494 29. Lõoke M, Kristjuhan K, Kristjuhan A. Extraction of genomic DNA from yeasts for PCR-based
495 applications. BioTechniques. 2011 May;50(5):325–8.

496 30. Tolner B, Smith L, Begent RHJ, Chester KA. Production of recombinant protein in Pichia pastoris
497 by fermentation. Nat Protoc. 2006 Aug;1(2):1006–21.

498

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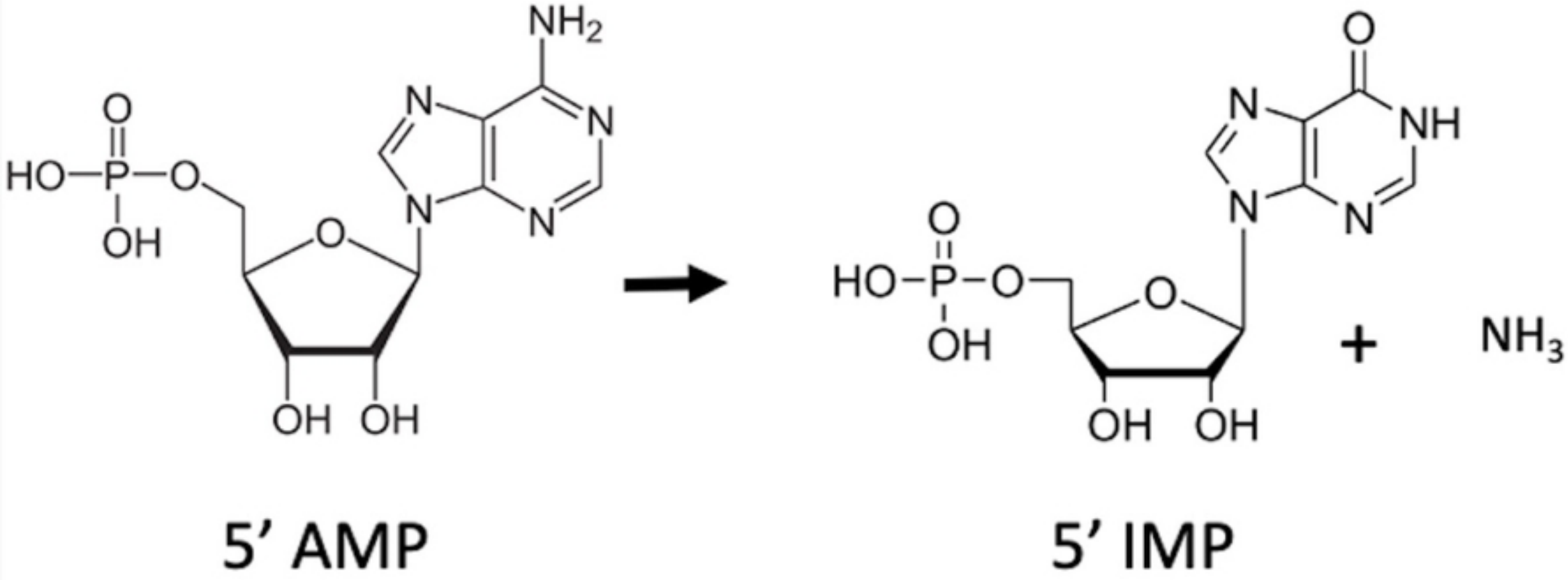

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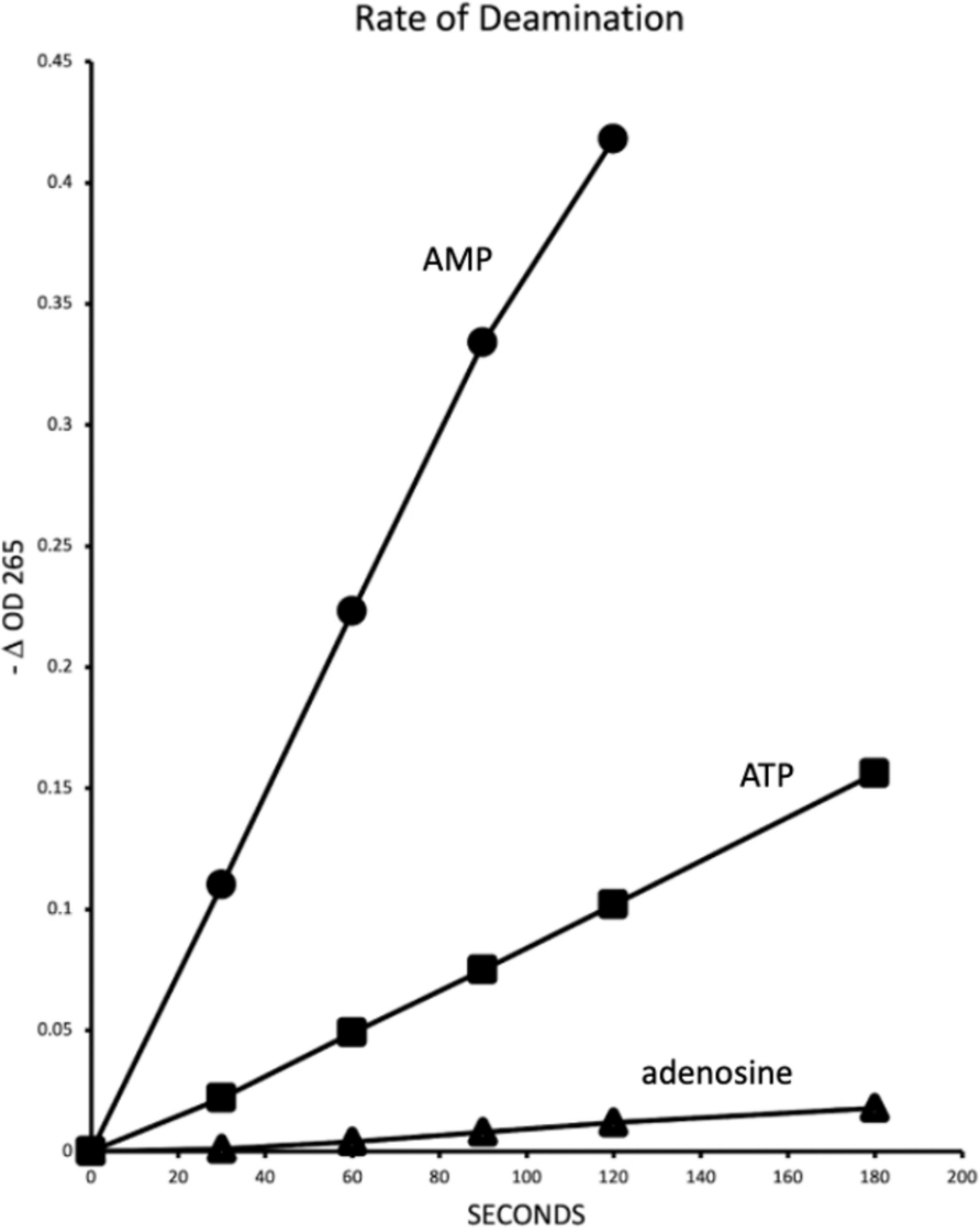
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0.3
0.25
0.2
0.15
0.1
0.05
0
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AMP
ATP
adenosine
0
20
40
60
80
100
120
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160
180
200
SECONDS

Figure

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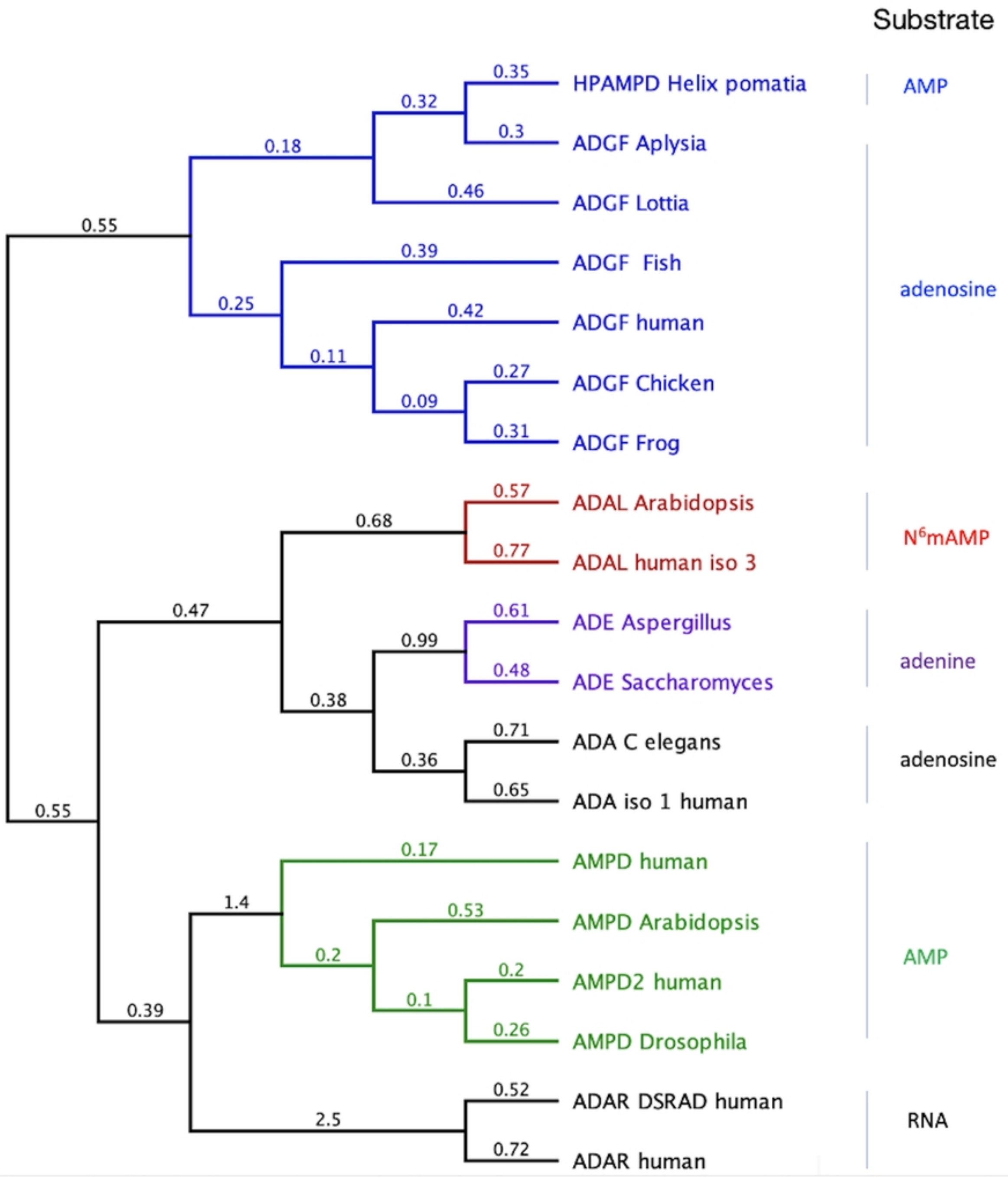
Figure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ADGF Aplysia
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ADGF Fish
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ADGF Frog
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ADE Aspergillus
ADE Saccharomyces
ADA C elegans
ADA iso 1 human
AMPD human
AMPD Arabidopsis
AMPD2 human
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ADAR DSRAD human
ADAR human
AMP
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adenosine
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Figure

```
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ADGF Aplysia californica           MSSFSTHNFVAIATFVCWFCCLATAAPLTSKAAYLLKRNSLIEEDASRKLGAK
MLGF Haliotus discus               MTYSCFKAYFPLLLLNVLFLHIDCMSISAYTLERQRIMKSEESDMFGSS
ADGF Lottia gigantea               MTKGLFIVFLTVWQWNYCVCVPASYQRQRDAIVQKEMDMRIGSH
ADGF Homo sapien                   MLVDGPSERPALCFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGR
ADGF Xenopus laevis                MACVSHWLLLLSLASATLSRPLWSERNGLIEMENSIRLGGN
ADGF Gallus gallus                 MKPPSGQGIRLACLLGLASLCLPTPLWEDRDSLMQEEEARLTGGN
ADGF Danio rerio                   MHVLFLGDLMWIYLLLLCCASCNGRPDPRKRDALIGLEASRRTGGD

                                   60        70        80        90       100       110
HPAMPD                             INLNNKEKKLNQFILREKRAIIENSRLNKTQYIPAVSFFLSKSQMESTPIFKIIKDM
ADGF Aplysia californica           IVLTNEEKVLDDFILAEKRKLIDDSRLNQTEYMPAASFYRSKDFIDTTFAYKIIQDM
MLGF Haliotus discus               LVLNKQEGLISDILIKAKRAEIEDSRVNGTIFPPAINFFESKKLIDSSKVFQIIKRM
ADGF Lottia gigantea               WNLTDDEKIVNNIFLGAKKNDYFNARVNNSFYPPAQNFFQSKKFIDKSLVFQMIEKM
ADGF Homo sapien                   LVLNTKEELANERLMTLKIAEMKEA-MRTLIFPPSMHFFQAKHLIERSQVFNILRMM
ADGF Xenopus laevis                IILTPSEATANQKLMTVKGAEFKEA-ESTGLFPPSMHFFKARPLIQQSHVFSILHQM
ADGF Gallus gallus                 LVLSTQEQQLSMKLISLKKKEVAAA-LNTGQFPPSMHFFRAKSLIEQSAVFSILKRM
ADGF Danio rerio                   ITLNEREKLLDGKLQKLKQHDMEA-----GQFPPSMHFFKAKRLIDQSPVFNLIQKM

                                  120       130       140       150       160       170
HPAMPD                             PKGAALHLHDTASARIDWIVSNATYRDHVYMCMDQ-DNFVRLTVSGTGP--PANSGC
ADGF Aplysia californica           PKGGALHLHDLAIASLDWVVKNATYRDNVYMCMDK-DNDVNLRVLQLIP--P-DPFC
MLGF Haliotus discus               PKGGALHTHGSAITSLDWLVKNVTYRANCYMCHDN-QGHLQMHFYDTPP--Q-PSYC
ADGF Lottia gigantea               PKGCALHIHDSSVASLDWFIQNVTYRDNLYICFNSVSGKPSLHFYSKAP--A-NPDC
ADGF Homo sapien                   PKGAALHLHDIGIVTMDWLVRNVTYRPHCHICFTP-RGIMQFRFAHPTP--RPSEKC
ADGF Xenopus laevis                PKGGALHLHDFAILSVDWLVKNASYMADCYMCLTR-DGGVRFLFAKPAPVGMLPPGC
ADGF Gallus gallus                 PKGAVLHLHDYAMLSVEWLVYNATYLPDCYICFTQ-TGTVRFRFSKPHPPHPVPAQC
ADGF Danio rerio                   PKGAALHVHDFAMVSVDWLVKNVTYRENCYVCFTD-KQTVQFIFSSGPP--ASSSHC

                                      180       190       200       210       220
HPAMPD                             -EWKLVETERANSGDIAAFDHWLKSNISLLTTDPLVTYPSLDKVWGRFDKHFSQLRG
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MLGF Haliotus discus               -QWESVQHVRERSGNALLFDQQLIKNISIIVDDPHSTYVTVNDVWRRFGLYFKIVNG
ADGF Lottia gigantea               -YWELISAVRKRSGDAKVFDEELYKNLSIIVDNPDDVYKTVDDVWQKFNDYFSLLNG
ADGF Homo sapien                   SKWILLEDYRKRVQNVTEFDDSLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISG
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ADGF Gallus gallus                 SQWVLLETYRKQLRNVTEFDHSLLRNLTLVTDAPELAYPSQAFIWKRFEEVFLIASG
ADGF Danio rerio                   SSWTLLRSLREKIKNTTELDNSFIRNLTLFTEDPDRAYPNQDTVWERFEQVFLVAYG

                                  230       240       250       260       270       280
HPAMPD                             IIYHTPIRRDYYRQILEEFRSDNVQYVEVRSSLSGYYDLDGTVHDPEYGLQLYKAVT
ADGF Aplysia californica           LLFYAPIMRDYYRQALEEFRADNVQYIELRSQLFGFFELDGTVHDAEFGLNLYKSVT
MLGF Haliotus discus               LYRYTPIFKDYLQQALREFEEDNVQYIEIRATLSPLYELDGSKHDQEHKLLTYRDTV
ADGF Lottia gigantea               AFQYVPVFQDYFRRALSEFYQDNVQCVELRVTLSPIYELDGSVKSPEWIVNAYRTIA
ADGF Homo sapien                   LIHYAPVFRDYVFRSMQEFYEDNVLYMEIRARLLPVYELSGEHHDEEWSVKTYQEVA
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ADGF Gallus gallus                 LICYAPVFKAYFYQGLLELYEDNIQYVEIRAILPAVYELDGTQHNKSWSVAAYQEVS
ADGF Danio rerio                   LVTYAPVFKDYLYEGLRQFYEDNIMYVEIRALLPQTYELDGRLNDKDWSMAACQEVV

                                  290       300       310       320       330       340
HPAMPD                             EEFVRTYPDFSGAKIIKSTARVK--PNTDIFNDVKLSMDLYKRYPGFFLGFDLVAQE
ADGF Aplysia californica           EEFQREYPDFIGAKIILSGLRFK--SQEEILNEVKIAMDLHKKYPDFFLGYDLVGQE
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ADGF Lottia gigantea               KEFIQQNPDFLGVKLIYSGIRYVFFPKPDILKDVKTAMSLIQKYKDFVVGFDLVGQE
ADGF Homo sapien                   QKFVETHPEFIGIKIIYSDHRSK--DVAVIAESIRMAMGLRIKFPTVVAGFDLVGHE
ADGF Xenopus laevis                NKFVGAHPDFLGAKIIYTVHRHE--DLAQVTEAVHLAMKLMEAFPEIMAGFDLVGQE
ADGF Gallus gallus                 RRFVQEHPDFLGAKVIFAAHRML--NVSQIKETIITAMELRAHFPDTLAGFDLVGNE
ADGF Danio rerio                   NQFKKHHPDFIGARVIFTVHRKI--NATEAVKTVEEAMILRRNFPDVMAGFDFVGQE

                                      350       360       370       380       390
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ADGF Lottia gigantea               GLGKPLLYYLDALLYPSQQSPPVNLPYFFHSGETKWQDTIVDYNLVDALLLNTTRIG
ADGF Homo sapien                   DTGHSLRDYKEALMIPAKDG--VKLPYFFHAGETDWQGTSIDRNILDALMLNTTRIG
ADGF Xenopus laevis                DAGHSLYQLSDALNIPSKLG--VKLPYFFHAGETNWQGKDVDENVLDALLLNTTRIG
ADGF Gallus gallus                 DEGHSLWEFKDVLTIPYSLR--ANLPYFFHAGETDWQGTSVDNNVLDALILNTSRIG
ADGF Danio rerio                   DLGRPLWYFKDALSLPEDKG--FNLPYFFHAGETDSQGTDVDQNLMDALLFNTTRIG

                                  400       410       420       430       440       450
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ADGF Lottia gigantea               HGFALTKHPYIKNMVEKRGIAVELNPISNQVLKLVDDIRNHPGAILMTKKFPVVVSS
ADGF Homo sapien                   HGFALSKHPAVRTYSWKKDIPIEVCPISNQVLKLVSDLRNHPVATLMATGHPMVISS
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ADGF Gallus gallus                 HGLALTKHPVAKNLSLKRDIPVEVCPISNQVLLLVSDLRSHPAASLMAEGHPVVVSP
ADGF Danio rerio                   HGFALARHPVVKEMARKMYVPIEVCPISNQVLKLVSDLRDHPAAVLMAEGHPLVISS

                                  460       470       480       490       500       510
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ADGF Gallus gallus                 DDPSIFGAKGVSYDFYEVFMAIGGMNADLRTLKQLALNSIKYSAMLLDEKAKAREVW
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                                  520       530       539
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ADGF Homo sapien                   KKRWDKFIADVATK
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ADGF Danio rerio                   LVKWDKFVWESLL
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Figure

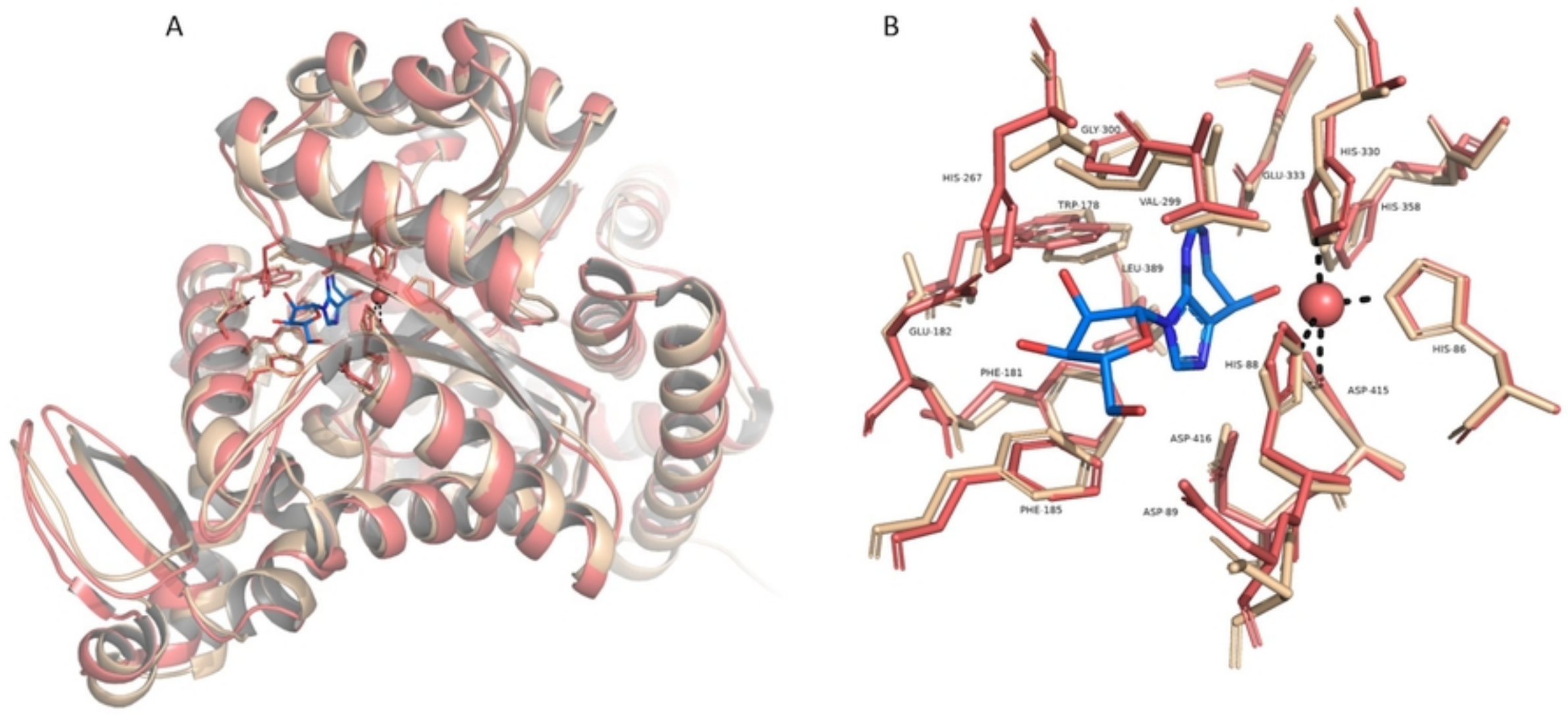
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Figure

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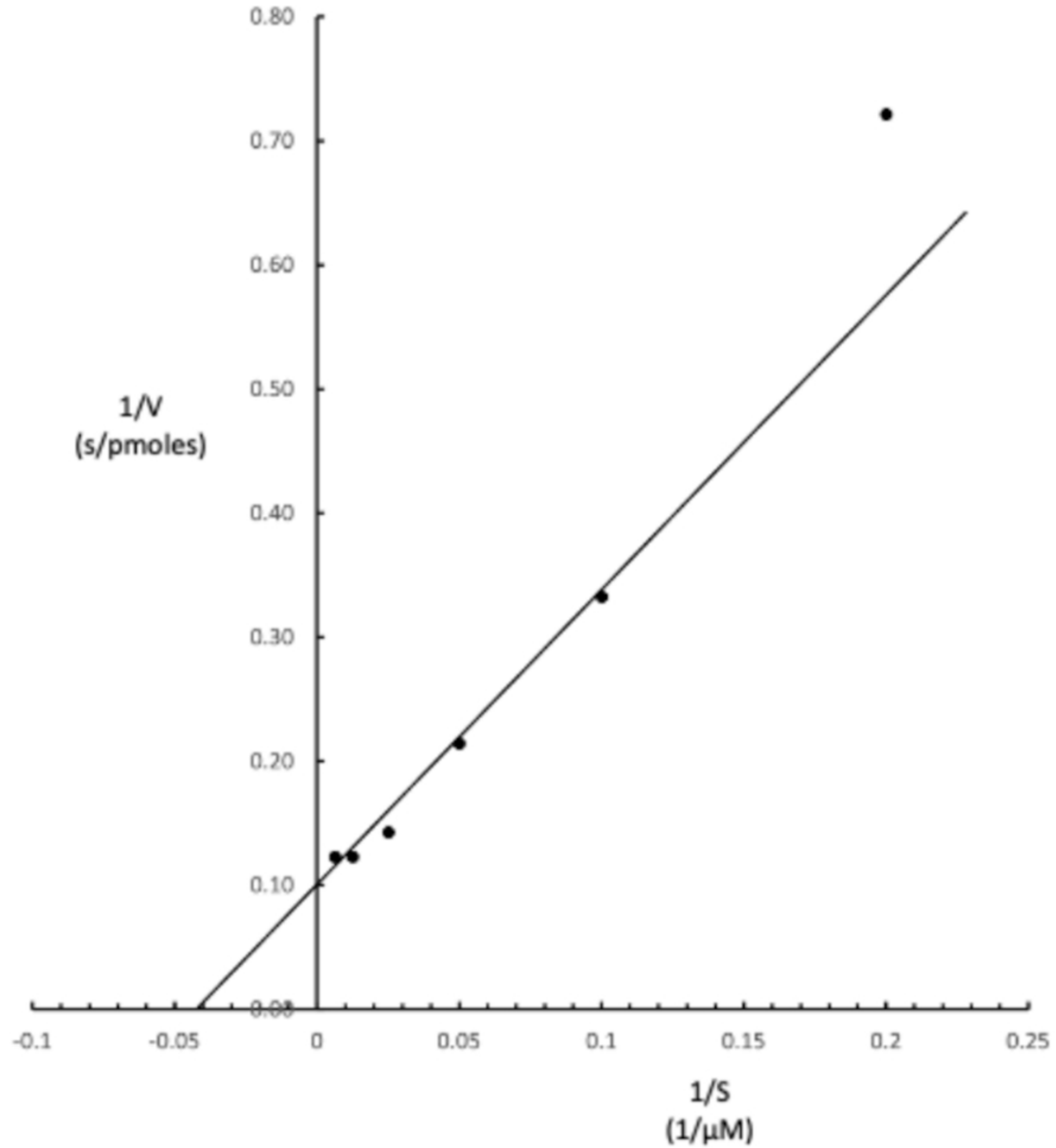
Figure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0
0.05
0.1
0.15
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1/S
(1/μM)

Figure