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Thermostable Adenosine 5'-Monophosphate Phosphorylase from *Thermococcus kodakarensis* forms catalytically active inclusion bodies

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Abstract

Catalytically active inclusion bodies (CatIBs) produced in *E. coli* are an interesting but currently underexplored strategy for enzyme immobilization. They can be purified easily and used directly as stable and reusable heterogenous catalysts. However, very few examples of CatIBs that are naturally formed during heterologous expression have been reported so far. Previous studies have revealed that the adenosine 5'-monophosphate phosphorylase of *Thermococcus kodakarensis* (*Tk*AMPpase) forms large soluble multimers with high thermal stability. Herein, we show that heat treatment of solubilized protein induces aggregation of active protein which phosphorolysis all natural 5'-mononucleotides. Additionally, inclusion bodies formed during the expression in *E. coli* were found to be similarly active with 2−6 folds higher specific activity compared to the heat-induced aggregates. Interestingly, differences in the substrate preference were observed. These results show that the recombinant thermostable *Tk*AMPpase is one of rare examples of naturally formed CatIBs.

Key words:

CatIB; heat induced aggregation; Thermostable AMP phosphorylase; Nucleoside monophosphate; phosphorolytic cleavage

List of abbreviations:

AMP, Adenosine 5'-monophosphate; *TK*AMPpase, *Thermococcus kodakarensis* Adenosine 5'-monophosphate phosphorylase; CatIB, catalytically active inclusion body; CMP, Cytosine 5'-monophosphate; GMP, guanosine 5'-monophosphate; HIA, Heat-induced aggregate; IB, Inclusion Body; NMP, Nucleoside monophosphate; R15P, Ribose-1,5-bisphosphate; UMP, Uridine 5'-monophosphate

Introduction

Heterologous expression of proteins in *Escherichia coli* often leads to aggregation of the target protein if its synthesis rate is higher than the folding rate. These intracellular aggregates, often called inclusion bodies (IBs), are typically composed of folding intermediates. IBs generally contain the aggregated protein in high concentration with relatively little contamination by other intracellular proteins. For this reason, intentional aggregation of the target protein and purification of the resulting IB is a widely used strategy for product concentration and crude purification in the pharmaceutical industry. Nonetheless, laborious downstream purification, solubilization and refolding steps are typically required to obtain the target protein in the correct (active) folding state.[1,2]

Catalytically active inclusion bodies (CatIBs) offer a valuable alternative as they can be produced in *E. coli* without the need for laborious solubilization. As such, CatIBs can be used as immobilized enzymes, thus, increasing the interest in this class of protein aggregates in recent years.[1,3,4] The application of CatIBs offers several advantages compared to soluble or synthetic immobilized enzymes. These include compatibility with aqueous and non-aqueous media, straightforward and cheap purification, reusability and no loss of activity due to immobilization.[4]

CatIBs are usually engineered by the addition of a small peptide tags or aggregation-inducing protein domains to a protein of interest. These folding active centers guide the protein to accumulate in inclusion bodies. While most publications so far describe such engineered protein variants forming CatIBs,[5–9] only a few examples of naturally occurring proteins yielding CatIBs during overexpression in *E. coli* have been reported.[10–13]

Herein, we describe the isolation of catalytically active inclusion bodies and heat-induced aggregates of *Thermococcus kodakarensis* adenosine 5'-monophosphate phosphorylase (*Tk*AMPpase). This enzyme was first described in 2007 as a biocatalyst involved in a previously undescribed metabolic pathway in archaea.[14–16] This pathway is involved in supplying the ribose moiety of 5'-mononucleotides (NMP) to the central carbon metabolic pathway(s).[14] *Tk*AMPpase phosphorolytically cleaves the *N*-glycosidic bond of NMP in the presence of inorganic phosphate yielding the corresponding nucleobase and ribose-1,5-bisphosphate (R15P; **Fig 1**).[14–16] The enzyme has previously been misannotated as a thymidine phosphorylase until Sato et al.[14] recognized its function in the AMP metabolic pathway in archaea. Although *Tk*AMPpase's primary structure is closely related to thymidine phosphorylases, it's quaternary structure was reported to form unusual multimers (>40-mers).[16] While we were studying this enzyme in more detail, we discovered that it is prone to aggregation both during expression and after exposure to heat which led us to develop a purification method to obtain catalytically active IBs of this protein. Interestingly, insoluble preparations of *Tk*AMPpase retained high activity with all natural 5'-mononucleotides, opening new avenues for the application of this enzyme as a heterogenous biocatalyst.

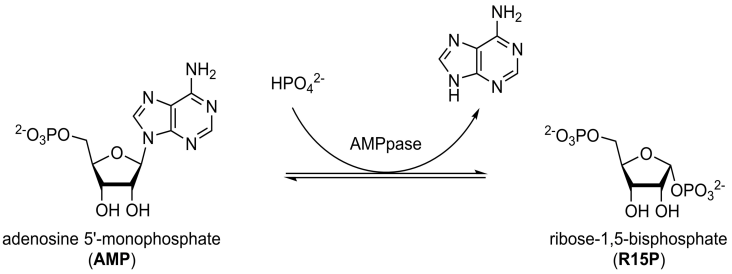

Figure 1 The reaction catalyzed by adenosine 5'-monophosphate phosphorylases (AMPpases). The N-glycosidic bond of adenosine 5'-monophosphate (AMP) is cleaved in the presence of inorganic phosphate yielding adenine and ribose-1,5-bisphosphate (R15P).

Materials and Methods:

Cloning and expression of *Tk*AMPpase

The *Thermococcus kodakarensis* gene (TK_RS01735) coding AMPpase (WP_011249307.1) was codon-optimized for expression in *E. coli* and obtained by gene synthesis (GeneArt™). The *Tk*AMPpase gene was then cloned via NdeI/HindIII digestion (FastDigest® restriction endonucleases, Fermentas, Vilnius, Lithuania) and ligation (T4 DNA Ligase, Roche) into a derivative of the expression vector pCTUT7 as described previously.[17] The *N*-terminally His_6-tagged *Tk*AMPpase was expressed in *E. coli* BL21 in 50 mL Enpresso B medium (Enpresso, Berlin, Germany) at 30 °C as recommended by the manufacturer. Expression was induced using IPTG with a final concentration of 1 mM.

An OD_{600} = 5 cell pellet was used to test the protein expression using BugBuster® Protein Extraction Reagent (Merck, Darmstadt, Germany) for cell lysis as recommended by the manufacturer. The cell lysate was centrifuged (4°C, 16,000 g for 15 min) to separate the soluble and the insoluble protein fractions. The protein fractions were analyzed using 12% SDS-polyacrylamide gels according to standard protocols[18] using a protein marker ranging from 10−200 kDa (New England BioLabs, MA, USA). The percentage of soluble and insoluble fractions was quantified from the SDS- polyacrylamide gels by densitometric analysis (ImageJ software (National Institute of Health, USA, http:// www.imagej.nih.gov/ij)).

Purification of *Tk*AMPpase as soluble protein

For cell disruption, 1 g of cell pellet was dissolved in 5 mL of 0.1 M Tris-HCl buffer containing 1 mM EDTA (pH 7). Cells were mechanically lysed by French Press to obtain soluble *Tk*AMPpase. The French press was used for five consecutive cycles at 900 bar. After each cycle, a sample of 1 mL was taken for further analysis. Following cell disruption, the suspension was treated with a solution of 1.5 M NaCl, 60 mM EDTA, 6% Triton-X100 (pH 7) and 0.1 mM PMSF (half of the remaining volume each) and incubated for 30 mins on ice. Insoluble protein was separated from

the solution by centrifugation (4°C, 16,000 g for 10 mins). Protein fractions (soluble and insoluble) were analyzed on 12% SDS- polyacrylamide gels and the soluble protein fractions were stored at 4°C for further use.

Aggregation of *Tk*AMPpase by heat

Protein aggregation was induced by heat treatment of the soluble *Tk*AMPpase at different temperatures (room temperature, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C). Samples were taken at 15, 30, 45 and 60 min and stored on ice. The heat-induced aggregates (HIAs) were collected by centrifugation (4°C, 13,000 g for 10 min). For SDS-PAGE analysis, 1:10 dilutions of HIAs were treated with standard SDS loading buffer containing 80 mM urea according to standard protocols.[18] Using densitometric analysis (ImageJ software (National Institute of Health, USA, http:// www.imagej.nih.gov/ij)), the relative amount of HIAs was calculated as a percentage of the total protein (soluble and insoluble) per time point. Standard deviations were calculated from three independent experiments. For analysis of the specific activity with 5'-mononucleotides, 0.2 g of HIAs were resuspended in 500 µl MOPS buffer (50 mM, pH 7.5).

Purification of CatIBs

For the isolation of CatIBs from *E. coli* cultures, 1 g of cell pellet was resuspended in 5 mL of 0.1 M Tris-HCl buffer (pH 7) containing 1 mM EDTA and 1.5 $mg.mL^{-1}$ lysozyme. The cell suspension was then incubated for 30 min at room temperature followed by mechanical lysis on ice using sonification for 5 min with 30 % power input and 30 sec on/off intervals. The mixture was then treated with DNase (final concentration of 50 µg) in the presence of 3 mM $MgCl_2$ and 0.1 mM PMSF. After incubation at 37°C for 30 min, a solution of 1.5 M NaCl, 60 mM EDTA and 6% Triton-X100 (pH 7) was added (half of the current volume). The cell suspension was then incubated for 30 min on ice. The IBs were collected by centrifugation (4°C, 8000 g for 10 min), followed by three successive washing steps each with 40 mL of 0.1 M Tris-HCl containing 20 mM EDTA (pH 7). The purified IBs were stored at 4°C until further use. For activity assays 0.2 g of isolated IBs were resuspended in 500 µL MOPS buffer (50 mM, pH 7.5).

Quantification of the *Tk*AMPpase

BSA was used as a standard to determine *Tk*AMPpase concentrations from different preparations (HIAs and IBs) using SDS-PAGE. Three different concentrations of BSA (0.5, 0.25 and 0.1 $mg.mL^{-1}$) were loaded on 12% SDS-polyacrylamide gels in duplicates. The concentrations were chosen to be in the linear detection range. The densities of the bands were analyzed using ImageJ software (National Institute of Health, USA, http:// www.imagej.nih.gov/ij) and standard curves were generated (**Supp. fig.1**). Serial dilution of the HIAs and CatIBs of *Tk*AMPpase were loaded on SDS- polyacrylamide gels and the band densities were quantified using the BSA standard curve as a reference (**Supp. fig. 2 and 3**).

Activity assay

The specific activity of the HIAs and CatIBs of *Tk*AMPpase were tested with natural NMPs. A proper dilution (1:32) of both preparations was used in which the enzyme OD_{260} did not exceed 0.1. Reactions were performed with 2 mM nucleotide and 50 mM phosphate in 50 mM MOPS buffer (pH 7) in the presence of 50−100 $\mu g\ mL^{-1}$ enzyme at 80°C. Regular samples were taken to monitor the initial rates of the reactions (<12% product formation). The minimum reaction time was 3 min for CatIBs and the maximum was 40 min for HIAs obtained at 90°C. Samples were stopped and quenched by adding 40 µL of reaction mixture to 460 µL of 0.5 M NaOH (CMP-containing reactions) or 0.1 M NaOH (AMP-, GMP- and UMP-containing reactions). The nucleobase/nucleotide ratio in each sample was obtained via deconvolution of the experimental UV absorption spectra using suitable reference spectra obtained under the same conditions as described previously.[19,20] The specific activities were calculated in units (U) per mg enzyme, where one U is the conversion of 1 µmol substrate per minute under the conditions stated above.

Results and Discussion

Expression and purification of *Tk*AMPpase as soluble protein

To study TkAMPpase in more detail, we expressed *Tk*AMPpase using an IPTG-inducible expression vector and a fed-batch medium (Enpresso B) in shake flasks. In contrast to previous reports,[15,16] recombinant *Tk*AMPpase (54 kDa) was expressed mainly in the insoluble fraction as detected in samples (OD_{600} = 5) taken after expression (**Fig. 2a**). On average about 80% of the totally expressed *Tk*AMPpase was found in the insoluble fraction as calculated using densitometric analysis. We attributed this to *Tk*AMPpase forming unusually large multimers (>40-mers).[16] This multimeric structure is linked together by 10 amino acid residues in the C-terminal domain. Additionally, the protein N-terminus (84 amino acid) contributes to the multimer formation. This multimeric structure of *Tk*AMPpase might explain the *in-vivo* aggregation of the heterologously expressed protein, although, it appears that expression conditions may lead to the formation of either insoluble (as in our study) or soluble aggregates or multimers as described previously.[15,16]

Since *Tk*AMPpase was obtained primarily as insoluble protein, we developed a high-pressure cell disruption protocol using a French press. Pressure of up to 2 kbar has previously been reported to disrupt the oligomerization of some proteins such as glyceraldhyde-3-phosphate dehydrogenase, lactate dehydrogenase, malate dehydrogenase or tryptophan synthase, which led us to hypothesize that a similar strategy might be employed to solubilize *Tk*AMPpase.[21,22] Indeed, after five consecutive cell disruption cycles at 900 bar, the amount of the recombinant *Tk*AMPpase in the soluble fraction increased from 30% to 80% with a stepwise increase after each cycle (**Fig. 2b, Supp. Fig 4**). One gram of wet cell pellet yielded around 5 mg of soluble *Tk*AMPpase as determined by densitometric analysis using BSA as a reference standard. These results suggested that the *Tk*AMPpase multimers do not withstand the high pressure during French Press cell disruption, forming smaller and better soluble units.

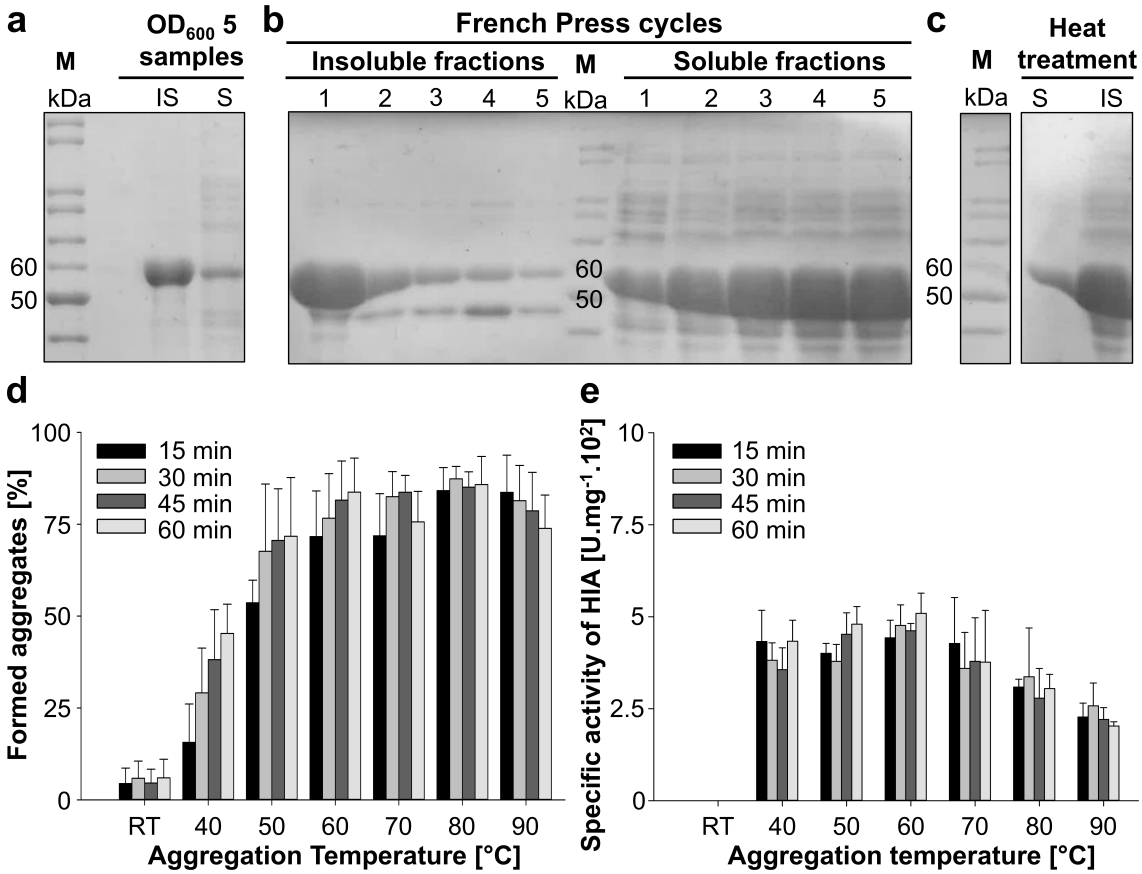

Figure *2*. Solubilization and formation of heat-induced aggregates (HIAs) of *Tk*AMPpase. (**a**) SDS-PAGE analysis of both soluble (S) and insoluble (IS) protein fractions of OD_{600}= 5 samples after the expression of *Tk*AMPpase in *E. coli* using Enpresso B media. The *Tk*AMPpase (54 kDa) is mainly detected in the insoluble fraction (IS) after cell disruption using BugBuster® Protein Extraction Reagent. M: protein marker. (**b**) Protein fractions obtained from French Press cell disruption at 900 bar analyzed by SDS-PAGE. (**c**) The fifth French Press fraction of the soluble protein was further purified by heat treatment at 80°C for 30 mins which resulted in a significant re-aggregation of the protein and its accumulation in the insoluble fraction. The marker (M) lane and the sample lanes are rearranged from the same gel. The complete SDS-PAGE from **a, b and c** are shown in the supplementary material. (**d**) The induction of aggregation by heat at different temperatures within 1 h. Room temperature was used as a negative control to examine spontaneous aggregation. Chart is generated based on densitometric analysis of SDS-polyacrylamide gels using ImageJ software. (**e**) The specific activity of all aggregates was determined with 2 mM CMP and 50 mM phosphate in 50 mM MOPS buffer (pH 7.5) at 80°C.

Formation and activity of heat-induced aggregates (HIAs)

*Tk*AMPpase is derived from a thermostable archaeon with an optimum growth temperature of 85°C.[23] Therefore, heat treatment is a valuable tool to purify *Tk*AMPpase. To explore this strategy, we subjected crude preparations of the soluble protein to 80°C for 30 min. In contrast to previous observations,[14] thermal treatment of the soluble protein led to almost complete precipitation of *Tk*AMPpase (**Fig. 2c**). To study this effect in more detail, the impact of temperature on the enzyme's solubility and the formation of aggregates was evaluated. Therefore, the soluble *Tk*AMPpase was incubated at six temperatures (from 40°C to 90°C in 10°C increments) and aggregate formation was assessed every 15 min for 1 h. Room temperature was used as a negative control to evaluate spontaneous aggregation and the relative amount of formed aggregates was calculated as a percentage of the total amount (soluble and insoluble) of the protein (**Fig. 2d, Supp. fig. 5**). At the lower temperatures tested (40°C, 50°C and 60°C), an increase of the percentage of aggregates over time was observed. While a steady increase corresponding to an aggregation rate of 0.84 h^{-1} was monitored at 40°C **(Supp. fig. 6)**, the aggregation at higher temperatures quickly reached a plateau of around 75% (50°C) and 80% (60°C). At even higher temperatures (70−90°C), 80% aggregation was already detectable after 15 min with no significant change within the remaining hour (**Fig. 2d**). The aggregation observed at room temperature was negligible (<5%) and did not increase over time. We attribute these observations to the formation of thermally stable multimers of *Tk*AMPpase which aggregate as insoluble proteins. Our results reveal that this aggregation happens at temperatures as low as 40°C with a highly temperature-dependent rate.

As it has been reported that *Tk*AMPpase multimers show activity towards a variety of 5'-mononucleotides,[16] we questioned if the formed HIAs retained phosphorolytic activity. Based on previously reported experimental data,[15] we used the phosphorolysis of CMP as a model reaction to determine the specific activity of the HIAs at 80°C. These experiments revealed that

all HIAs have catalytic activity with values of 200 − 500 U.mg^{-1} (**Fig. 2e**). The HIAs obtained through aggregation between 40°C and 60°C showed no significant differences in their specific activities. In contrast, the aggregates formed at temperatures between 70°C and 90°C displayed a gradual decrease in their specific activity with the lowest activity observed after aggregation at 90°C (**Fig. 2e, Supp. Fig 7**). These data suggest that with increasing temperature partial denaturation of *Tk*AMPpase occurs. However, full denaturation of *Tk*AMPpase was not observed under the applied conditions and all HIA preparations retained activity.

Purification of CatIBs

Since *Tk*AMPpase is mainly expressed in the insoluble fraction and even aggregated protein showed activity in a model reaction, IB isolation seemed as an attractive alternative for protein purification. Therefore, we attempted to isolate active IBs of *Tk*AMPpase after expression in *E. coli*. To avoid the disruption of the *in-vivo* formed IB, sonication was used as a gentler cell disruption method compared to the French Press treatment applied before. This method yielded intact *in-vivo* formed IBs which were successfully purified directly from the insoluble protein fraction (**Fig. 3a, 3b**). Densitometric analysis using BSA as a reference revealed that 1 g of wet cell pellet yielded approximately 0.5 g of IBs (wet weight; **Supp. Fig 3**).

Specific activity of *Tk*AMPpase

Next, we investigated if these purified IBs of *Tk*AMPpase displayed activity comparable to that of HIAs formed at 60°C after 1h (**Fig 3c**). Thus, we assessed the phosphorolytic activity of both preparations (IBs and HIAs) with AMP, GMP, CMP and UMP in the presence of phosphate at 80°C. Our results revealed that the isolated IBs showed 2−6 fold higher specific activities towards all substrates compared to the HIAs (**Fig 3c**). Additionally, our CatIBs displayed one to two magnitudes higher specific activities compared to the soluble *Tk*AMPpase reported previously (**Fig 3d**).[15] This might be explained by differences in the applied reaction conditions as well as the purification procedure. We used a high ratio of phosphate to substrate (25:1), whereas, previous

reactions were carried out with equimolar phosphate and substrate.[15] The high phosphate concentration might drive the reaction towards the 5'-mononucleotides cleavage and lead to higher specific activities, especially considering the previously reported equilibrium constant of phosphorolysis of AMP of 0.006.[14]

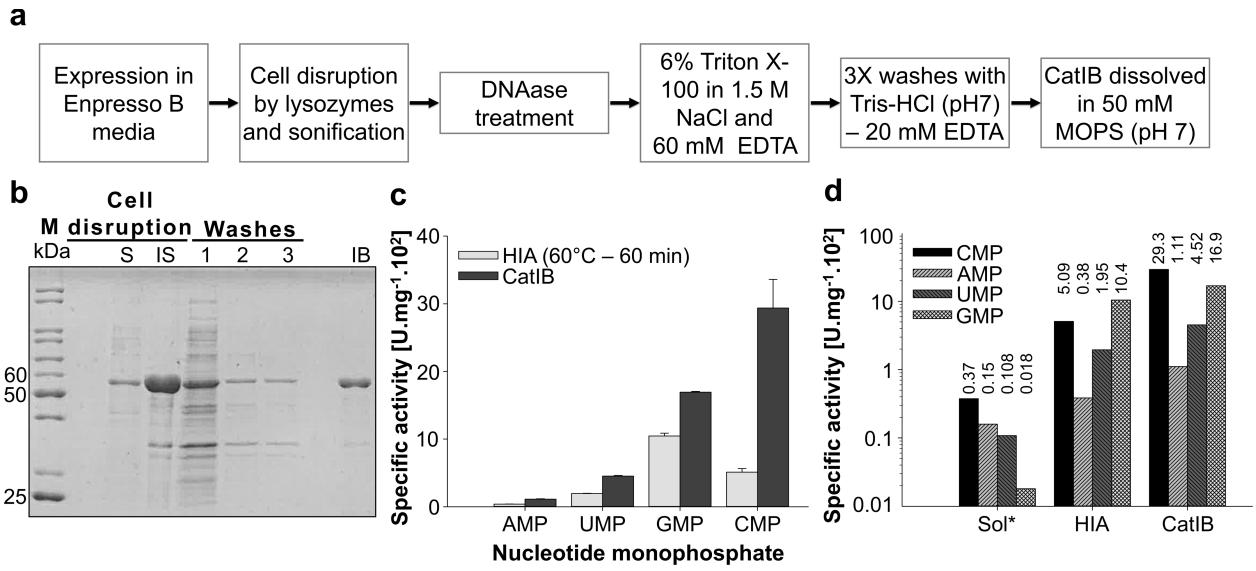

Figure 3 Expression and purification of CatIBs. (a) The utilized protocol for IB isolation. **(b)** 12% SDS-polyacrylamide gels showing the different steps of IB isolation. After sonification *Tk*AMPpase was expressed mainly in the insoluble fraction (IS). Washing steps with Tris-HCl buffer were applied to decrease protein background. The final IB preparation showed only minor impurities (IB). **(c)** Specific activity of the CatIBs compared to the HIAs obtained from 60°C heat treatment after 60 mins was determined. The used reaction conditions were 2 mM substrate (NMP) and 50 mM potassium phosphate in MOPS buffer (pH 7) at 80°C. Regular samples were taken at different time points over a period of 15 min. Standard deviation is derived from three experiments. **(d)** Substrate preference of the different enzyme preparations; heat induced aggregates (HIA) and catalytically active inclusion bodies (CatIBs) reactions were performed as mentioned above. *Specific activity of the soluble protein (Sol.) is obtained from Aono et al. (2020).[15] Reaction conditions were 20 mM NMP and 20 mM sodium phosphate in 100 mM Tris-HCl (pH 7.5) at 85°C. M: protein ladder, S: soluble protein fraction after sonification.

Interestingly, a comparison between the activities of different enzyme preparations (HIA, CatIB and published soluble enzyme[15]) revealed a difference in the substrate preferences. The CatIBs showed the highest activity with CMP, while GMP is the preferred substrate for HIAs (**Fig. 3d**).

Altered substrate preferences is additionally observed as compared to published activity of the soluble enzyme (**Fig. 3d**).[15] There are increasing evidences that aggregates formed under different conditions (including different temperatures) display morphological differences,[24–26] with differences in secondary and tertiary structure additionally altering their function.[25] Since rather little is known about AMPpases, this class of enzymes requires further studies to create a better understanding of their structure function relationships and aggregation behavior.

Conclusion

*Tk*AMPpase belongs to the small group of enzymes that naturally forms CatIBs without an artificial tag during its heterologous expression in *E. coli*. Whereas high pressures achieve solubilization of the obtained insoluble protein, heat treatment at various temperatures induces the re-formation of insoluble aggregates. Interestingly, both the *in-vivo* formed IB and the *in-vitro* formed aggregates have catalytic activity and perform the phosphorolysis of natural 5'-mononucleotides. Although further work is necessary to gain a better understanding of the substrate spectrum of *Tk*AMPpase as well as the reasons for the observed aggregation, the results presented in this study encourage exploration of this enzyme as a self-immobilizing biocatalyst for applications in heterogenous reaction systems.

Author Contributions: Conceptualization, AK and SK; methodology, MCW; Data analysis, MCW, SK, SW, FK; writing—original draft preparation, SK and MCW; writing—review and editing, All authors; supervision, PN, AK and SK; project administration, PN, and AK; funding acquisition, SK, AK. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

AK is CEO and PN is a member of the advisory board of BioNukleo GmbH. MCW is a student worker, FK and SW are, and SK was a scientific researcher at the biotech company BioNukleo GmbH. The authors have no other relevant affiliations or financial interests in or financial conflicts with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Figures

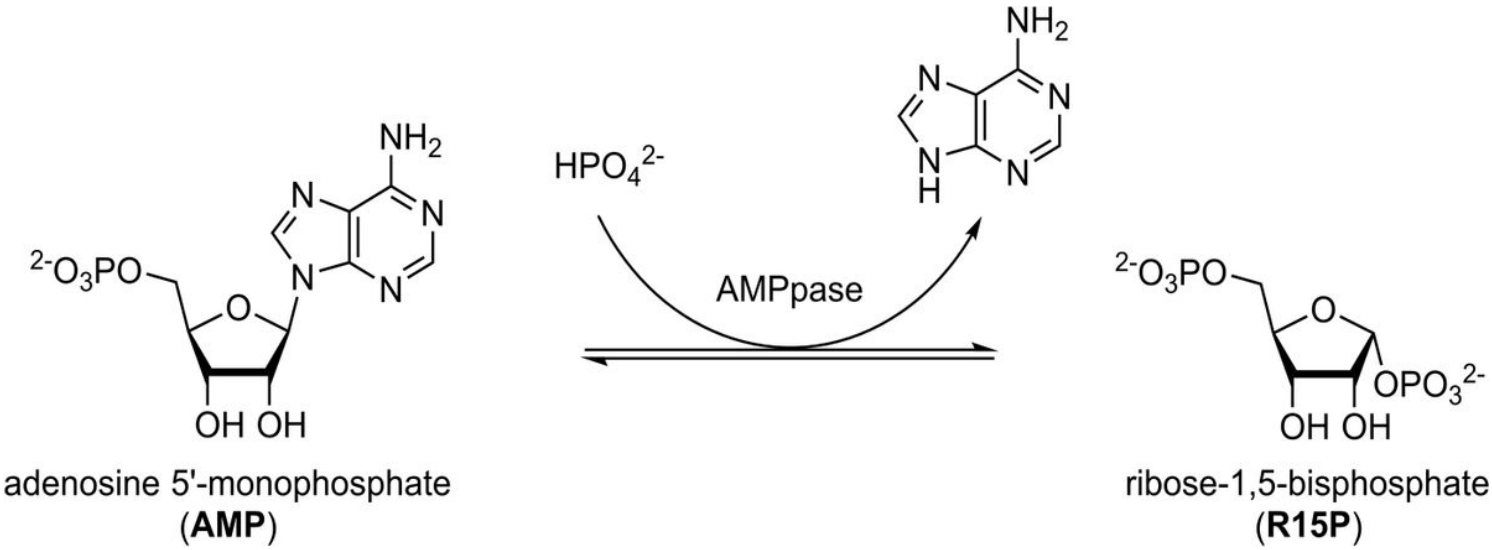

Figure 1

The reaction catalyzed by adenosine 5’-monophosphate phosphorylases (AMPpases). The N-glycosidic bond of adenosine 5’-monophosphate (AMP) is cleaved in the presence of inorganic phosphate yielding adenine and ribose-1,5-bisphosphate (R15P).

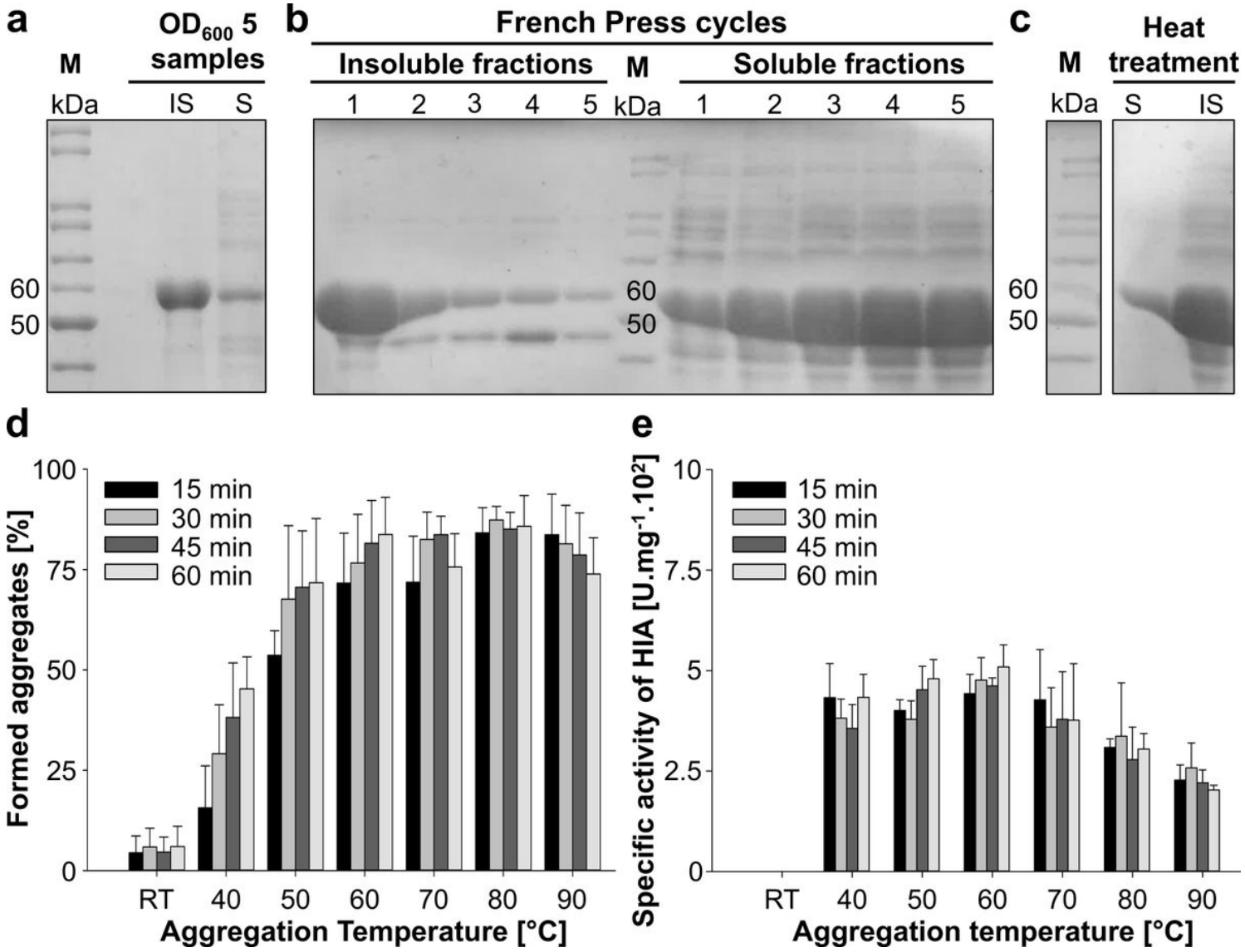

Figure 2

Solubilization and formation of heat-induced aggregates (HIAs) of TkAMPpase. (a) SDS209 PAGE analysis of both soluble (S) and insoluble (IS) protein fractions of OD600= 5 samples after the expression of TkAMPpase in E. coli using Enpresso B media. The TkAMPpase (54 kDa) is mainly detected in the insoluble fraction (IS) after cell disruption using BugBuster® Protein Extraction Reagent. M: protein marker. (b) Protein fractions obtained from French Press cell disruption at 900 bar analyzed by SDS-PAGE. (c) The fifth French Press fraction of the soluble protein was further purified by heat treatment at 80°C for 30 mins which resulted in a significant re-aggregation of the protein and its accumulation in the insoluble fraction. The marker (M) lane and the sample lanes are rearranged from the same gel. The complete SDS-PAGE from a, b and c are shown in the supplementary material. (d) The induction of aggregation by heat at different temperatures within 1 h. Room temperature was used as a negative control to examine spontaneous aggregation. Chart is generated based on densitometric analysis of SDS-polyacrylamide gels using ImageJ software. (e) The specific activity of all aggregates was determined with 2 mM CMP and 50 mM phosphate in 50 mM MOPS buffer (pH 7.5) at 80°C.

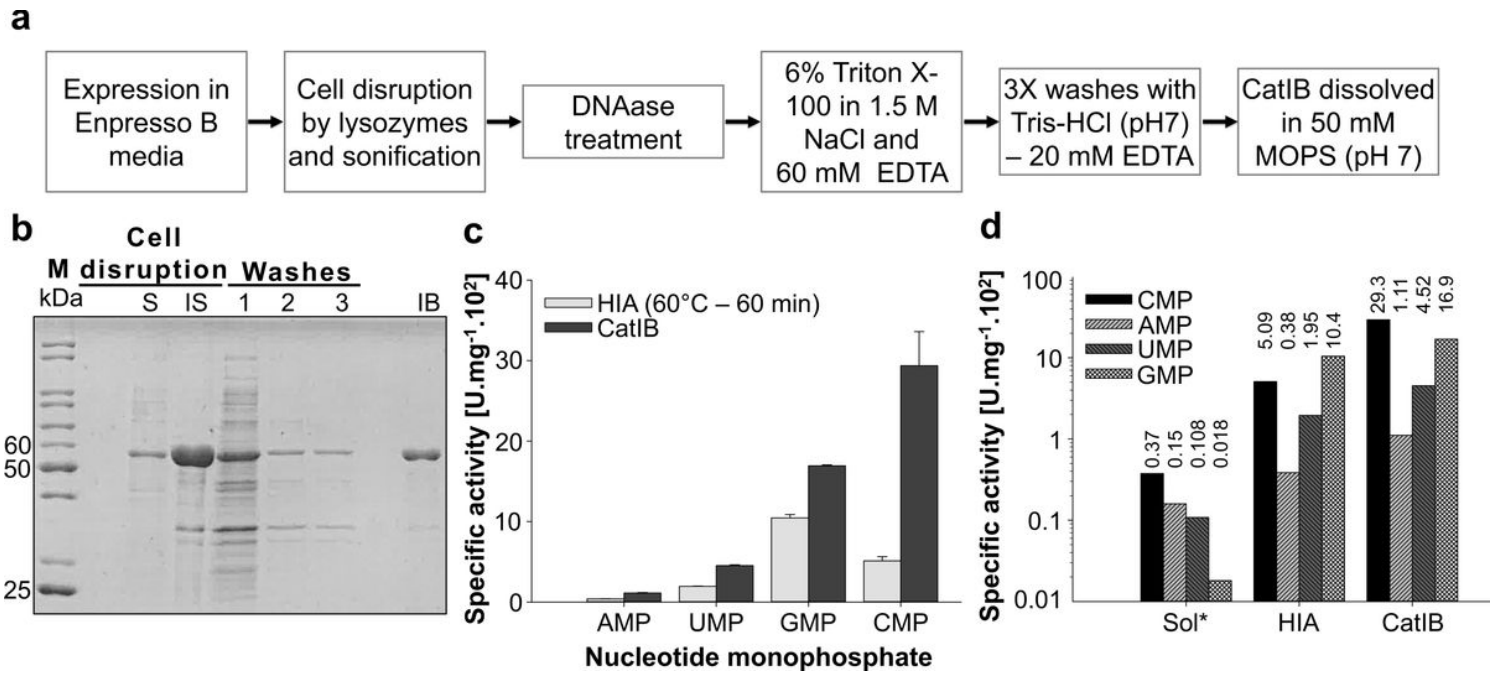

Figure 3

Expression and purification of CatIBs. (a) The utilized protocol for IB isolation. (b) 12% SDS278 polyacrylamide gels showing the different steps of IB isolation. After sonification TkAMPpase was expressed mainly in the insoluble fraction (IS). Washing steps with Tris-HCl buffer were applied to decrease protein background. The final IB preparation showed only minor impurities (IB). (c) Specific activity of the CatIBs compared to the HIAs obtained from 60°C heat treatment after 60 mins was determined. The used reaction conditions were 2 mM substrate (NMP) and 50 mM potassium phosphate in MOPS buffer (pH 7) at 80°C. Regular samples were taken at different time points over a period of 15 min. Standard deviation is derived from three experiments. (d) Substrate preference of the different enzyme preparations; heat induced aggregates (HIA) and catalytically active inclusion bodies (CatIBs) reactions were performed as mentioned above. *Specific activity of the soluble protein (Sol.) is obtained from Aono et al. (2020).15 Reaction conditions were 20 mM NMP and 20 mM sodium phosphate in 100 mM Tris-HCl (pH 7.5) at 85°C. M: protein ladder, S: soluble protein fraction after sonification.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Kameletal2020SupplementaryMaterial.pdf