Kinetics and structure of a cold-adapted hetero-octameric ATP phosphoribosyltransferase[†]

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Abbreviations: ATP, adenosine 5'-triphosphate; ATPPRT, ATP phosphoribosyltransferase; PRPP, 5-phospho- α -D-ribose-1-pyrophosphate; PR-ATP, N^1 -(5-phospho- β -D-ribose)-ATP; PP_i, inorganic pyrophosphate; *Ll*ATPPRT, *L. lactis* ATPPRT; HisRS, histidyl-tRNA synthetase; *Tm*ATPPRT, *T. maritima* ATPPRT; *Ll*HisGs, *L. lactis* HisGs; *Ll*HisZ, *L. lactis* HisZ; *Pa*ATPPRT, *P. arcticus* ATPPRT; ³¹P-NMR, ³¹P-nuclear magnetic resonance; CD, circular dichroism; HEPES, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid; DTT, dithiothreitol; *Pa*HisGs, *P. arcticus* HisGs; *Pa*HisZ, *P. arcticus* HisZ; IPTG, isopropyl- β -D-1-thiogalactopyranoside; LB, Lysogeny broth; TEVP, tobacco etch virus protease; *Mt*PPase, *Mycobacterium tuberculosis* inorganic pyrophosphatase; CV, column volumes; MWCO, molecular weight cut off; ESI-MS, electrospray ionisation mass spectrometry; K_D , equilibrium dissociation constant; *Ec*ATPPRT, *E. coli* ATPPRT; rmsd, root-mean square deviation.

Abstract: ATP-phosphoribosyltransferase (ATPPRT) catalyses the first step in histidine biosynthesis, the condensation of ATP and 5-phospho- α -D-ribosyl-1-pyrophosphate to generate N^1 -(5-phospho- β -D-ribosyl)-ATP and inorganic pyrophosphate. The enzyme is allosterically inhibited by histidine. Two forms of ATPPRT, encoded by the *hisG* gene, exist in nature, depending on the species. The long form, HisG_L, is a single polypeptide chain with catalytic and regulatory domains. The short form, HisG_S, lacks a regulatory domain, and cannot bind histidine. HisG_S instead is found in complex with a regulatory protein, HisZ, constituting the ATPPRT holoenzyme. HisZ triggers HisG_S catalytic activity while rendering it sensitive to allosteric inhibition by histidine. Until recently, HisG_S was thought to be catalytically inactive without HisZ. Here, recombinant HisG_S and HisZ from the psychrophilic bacterium *Psychrobacter arcticus* were independently overexpressed and purified. The crystal structure of *P. arcticus* ATPPRT was solved at 2.34-Å resolution, revealing an equimolar HisG_S-HisZ hetero-octamer. Steady-state kinetics indicate that both ATPPRT holoenzyme and HisG_S are catalytically active. Surprisingly, HisZ confers only a modest 2- to 4-fold increase in k_{cat} . Reaction profiles for both enzymes are indistinguishable by ^{31}P -NMR, indicating that the same reaction is catalysed. Temperature dependence of k_{cat} shows deviation from Arrhenius behaviour at 308 K with the holoenzyme. Interestingly, such deviation is detected only at 313 K with HisG_S. Thermal denaturation by CD spectroscopy resulted in T_{m} 's of 312 K and 316 K for HisZ and HisG_S, respectively, suggesting that HisZ renders the ATPPRT complex more thermolabile. This is the first characterisation of a psychrophilic ATPPRT.

Adenosine 5'-triphosphate phosphoribosyltransferase (ATPPRT) (EC 2.4.2.17) catalyses the reversible Mg^{2+} -dependent reaction between adenosine 5'-triphosphate (ATP) and 5-phospho- α -D-ribosyl-1-pyrophosphate (PRPP) to yield N^1 -(5-phospho- β -D-ribosyl)-ATP (PR-ATP) and inorganic pyrophosphate (PP_i) (Scheme 1), the first step in histidine biosynthesis.¹ The chemical equilibrium of the reaction strongly favours reactants,² and the enzyme is allosterically inhibited by histidine.¹ In addition to being a model for understanding allostery,²⁻⁴ ATPPRT is of biotechnological interest as a tool for histidine production, provided that histidine feedback inhibition can be overcome.⁵⁻⁷

Two forms of ATPPRT, encoded by the *hisG* gene, are found in nature. Fungi, plants, and most bacteria possess a long, homo-hexameric protein, HisG_L, each subunit consisting of two domains that make up the catalytic core and a C-terminal regulatory domain that mediates feedback inhibition by histidine.⁸ Some bacteria and archaea have a short version of the protein, HisG_S, which lacks the C-terminal regulatory domain and is insensitive to histidine. In these organisms, a catalytically inactive regulatory protein, HisZ, the product of the *hisZ* gene, is present.⁹ HisZ, which shares a common ancestry with histidyl-tRNA synthetase (HisRS), binds HisG_S to form the hetero-octameric ATPPRT holoenzyme and activates HisG_S catalysis.^{10, 11} Furthermore, HisZ binds histidine to allosterically inhibit the holoenzyme.^{11, 12}

While much is known about the mechanism,¹³⁻¹⁸ structure,^{4, 8, 19, 20} and regulation^{3-7, 15} of HisG_L-type ATPPRT's from several organisms, limited information is available on HisG_S-HisZ-type ATPPRT's, with the enzymes of only two species, the mesophilic bacterium *Lactococcus lactis* (LlATPPRT),^{10, 11, 21} and the thermophile *Thermotoga maritima* (TmATPPRT),¹² being reported. Only recently was it demonstrated, contrary to previous assumption, that *L. lactis* HisG_S (LlHisG_S) retains some catalytic activity in the absence of *L. lactis* HisZ (LlHisZ).²² Likewise, while mesophilic and thermophilic ATPPRT's have been characterised,^{11, 12} no psychrophilic example has been reported.

Psychrophilic enzymes have maximum activities at lower temperatures than those of their mesophilic and thermophilic counterparts, and also present higher protein flexibility.²³⁻²⁶ Thought to be an adaptation to support catalysis at low temperature, this increased flexibility also results in thermolabile proteins that tend to unfold at relatively mild temperatures.^{27, 28} Owing to their peculiar properties, investigations of cold-adapted enzymes have an impact on physical chemistry,^{25, 26, 29} biotechnology,^{30, 31} and even astrobiology.^{32, 33}

In the present work, the first characterisation of a psychrophilic ATPPRT is described. Recombinant HisGs and HisZ from the Siberian permafrost bacterium *Psychrobacter arcticus*, which naturally grows at temperatures between -10 and 0 °C,³⁴ were overexpressed and purified, and X-ray crystallography, steady-state kinetics, and ^{31}P -nuclear magnetic resonance (^{31}P -NMR) and circular dichroism (CD) spectroscopies were employed to investigate the structural, catalytic, and temperature-dependent properties of *P. arcticus* ATPPRT (*Pa*ATPPRT). The initialism *Pa*ATPPRT henceforth refers to the holoenzyme consisting of the complex between catalytic and regulatory subunits.

EXPERIMENTAL PROCEDURES

Materials. ATP, PRPP, Glutaraldehyde, deuterium oxide (99.9 atom % deuterium), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), tricine, dithiothreitol (DTT), imidazole, glycerol, lysozyme, DNase I, ampicillin, kanamycin, and chloramphenicol were purchased from Sigma-Aldrich. EDTA-free Cømplete protease inhibitor cocktail was from Roche. Isopropyl- β -D-1-thiogalactopyranoside (IPTG) were purchased from Fisher Scientific. All other chemicals were purchased from readily available commercial sources, and all chemicals were used without further purification.

Expression of *P. arcticus* HisGs (*Pa*HisGs) and *P. arcticus* HisZ (*Pa*HisZ). The expression vectors pJexpress431 and pJexpress414 containing the DNA sequences encoding

for *PaHisGs* and *PaHisZ*, respectively, each with a tobacco etch virus protease (TEVP)-cleavable N-terminal His-tag-encoding sequence, all codon-optimized for heterologous expression in *Escherichia coli*, were obtained from DNA 2.0, Inc. The expression construct for *PaHisGs* was transformed into *E. coli* C43(DE3) competent cells (Sigma-Aldrich), whereas that for *PaHisZ*, into *E. coli* BL21(DE3) competent cells (Novagen). Transformed cells were grown independently in lysogeny broth (LB), containing either 50 $\mu\text{g mL}^{-1}$ kanamycin (for *PaHisGs* expression) or 100 $\mu\text{g mL}^{-1}$ ampicillin (for *PaHisZ* expression), at 37 °C to an $\text{OD}_{600\text{nm}}$ of 0.8. The cultures were then equilibrated to 16 °C and expression was induced with 1 mM IPTG. Cells were allowed to grow for an additional 20-h period at 16 °C, then harvested by centrifugation at 6,774 *g* for 15 min and stored at –20 °C.

Expression of TEVP and Mycobacterium tuberculosis inorganic pyrophosphatase (MtPPase). The expression vector pRK793 harbouring the coding sequence for an N-terminal His-tagged S219V-TEVP was transformed into *E. coli* Rosetta(DE3) competent cells (Novagen) and TEVP was expressed as previously published.³⁵ The expression vector pJexpress411 harbouring the coding sequence for an N-terminal His-tagged MtPPase (a kind gift from Dr Luiz Pedro S. de Carvalho, The Francis Crick Institute) was transformed into *E. coli* BL21(DE3) and expressed as previously described.³

Purification of PaHisGs and PaHisZ. Each recombinant protein was purified separately from the other, and all purification procedures were carried out at 4 °C. All chromatographic steps employed an AKTA Start FPLC system (GE Lifesciences). Cells were allowed to thaw on ice for 20 min before being re-suspended in buffer A (50 mM HEPES, 10 mM imidazole, 500 mM NaCl, pH 8.0) containing 0.2 mg mL^{-1} lysozyme, 0.05 mg mL^{-1} DNase I, and half a tablet of EDTA-free Cømplete protease inhibitor cocktail, disrupted in a high pressure cell disruptor (Constant Systems), and centrifuged at 48,000 *g* for 30 min to remove cell debris.

The supernatants were filtered through 0.45 μm membranes and loaded onto a HisTrap FF 5 mL column (GE Healthcare) pre-equilibrated with buffer A. The column was washed with 10 column volumes (CV) of buffer A, and the adsorbed proteins were eluted with 20 CV of a linear gradient of 0% to 60% buffer B (50 mM HEPES, 500 mM imidazole, 500 mM NaCl, pH 8.0). Fractions containing the desired protein were pooled, mixed with TEVP at a ratio of either 1 mg of TEVP to 10 mg of *PaHisGs* or 1.5 mg of TEVP to 10 mg of *PaHisZ*, and dialysed twice against 2 L of buffer C (20 mM HEPES, 150 mM NaCl, 2 mM DTT, 10% glycerol, pH 7.5) then once against 2 L of buffer A. Samples were filtered through 0.45 μm membranes and loaded onto a HisTrap FF 5 mL column (GE Healthcare) pre-equilibrated with buffer A. The flow through was collected and analysed by SDS-PAGE (NuPAGE Bis-Tris 4-12% Pre-cast gels, ThermoFisher Scientific), concentrated using 10,000 molecular weight cut off (MWCO) ultrafiltration membranes (Millipore), dialysed twice against 2 L of 20 mM HEPES pH 8.0, aliquoted, and stored at $-80\text{ }^{\circ}\text{C}$. Concentrations of *PaHisGs* and *PaHisZ* were determined spectrophotometrically (NanoDrop) at 280 nm using theoretical extinction coefficients ($\epsilon_{280\text{nm}}$) of $8,940\text{ M}^{-1}\text{ cm}^{-1}$ and $25,900\text{ M}^{-1}\text{ cm}^{-1}$, respectively. Each protein's molecular mass was determined by electrospray ionisation mass spectrometry (ESI-MS).

Purification of TEVP and MtPPase. All purification steps were carried out at $4\text{ }^{\circ}\text{C}$, and all chromatographic steps employed an AKTA Start FPLC system. TEVP was prepared as previously published³⁵ and stored at $-80\text{ }^{\circ}\text{C}$. *MtPPase* was purified in the same manner as *PaHisHs* and *PaHisZ* up to and including the first HisTrap FF 5 mL column. Fractions containing *MtPPase* were pooled, concentrated using a 10,000 MWCO ultrafiltration membranes and loaded onto a HiPrep 26/60 Sephacryl S-300 HR column pre-equilibrated with 20 mM HEPES pH 8.0. *MtPPase* was eluted with 1 CV of 20 mM HEPES pH 8.0, analysed by SDS-PAGE, concentrated using a 10,000 MWCO ultrafiltration membrane, aliquoted, and

stored at -80°C . *MtPPase* concentration was determined spectrophotometrically at 280 nm using an $\epsilon_{280\text{nm}}$ of $19,940\text{ M}^{-1}\text{ cm}^{-1}$.

Crystallisation of PaATPPRT. Initial sparse matrix crystallisation screening was performed in 96-well Intelli-Plates using a Gryphon crystallization robot (Art Robbins Instruments), and hits visualized on a Rigaku Minstrel HT UV. The initial hit (15.73% PEG 3350, 0.1 M bicine pH 8.5, 0.12 M CaCl_2 , and 2.16% 1,6-hexanediol) was optimized to 11% PEG 3350, 0.1 M bicine pH 8.5, 0.15 M SrCl_2 , 0.15 M KBr, and 2% 1,6-hexanediol. *PaHisGs* and *PaHisZ* were mixed in 1:1 molar ratio, the buffer was exchanged to 0.02 M Tris pH 7.0, 0.05 M KCl, 0.01 M MgCl_2 , 2 mM DTT, 0.5 mM histidine, and 10 mM ATP. *PaATPPRT* was concentrated to 8 mg mL^{-1} . Using the hanging-drop vapour-diffusion method in 24-well plates with protein and precipitant mixed in a 1:1 ratio, crystals ($0.3\text{ mm} \times 0.1\text{ mm} \times 0.1\text{ mm}$) grew for 21 days at 277 K.

X-ray data collection and processing. Crystals were cross-linked by vapour diffusion with 25% (v/v) aqueous solution of glutaraldehyde for 1 h prior to being cryoprotected in 15% 2,4-methylpentanediol freshly prepared in reservoir solution, then flash-cooled in liquid nitrogen. Diffraction data were collected on a Rigaku 007 MM HFM generator with a Saturn 944+ CCD detector. Data were processed with iMosflm³⁶ and scaled with Aimless.³⁷ Matthews coefficient³⁸ calculations indicated that the asymmetric unit contained an equimolar heterooctamer. The structure was solved using molecular replacement as implemented in Phaser³⁹ and a combination of three sets of deposited coordinates as search models: PDP ID's 1Z7M (*LiATPPRT*),⁴⁰ 2VD2 (*B. subtilis* HisG, unpublished), and 3OD1 (*B. halodurans*, unpublished). CCP4 Buccaneer^{41, 42} was used to assign the majority of correct side chains to the *PaATPPRT* model. Missing side chains and residues were added with COOT⁴³ and the model refined with Refmac.⁴⁴ Secondary structures were assigned with STRIDE.⁴⁵ Inter-subunit interface parameters were calculated with PISA.⁴⁶

PaATPPRT and PaHisG_S activity assays. All assays were performed under initial rate conditions in the forward direction in 100 mM tricine pH 8.5, 15 mM MgCl₂, 100 mM KCl, 4 mM DTT, and 10 μ M *MtPPase*, at different constant temperatures, unless otherwise stated. *MtPPase* irreversibly hydrolyses PP_i into two equivalents of inorganic phosphate, overcoming the unfavourable reaction equilibrium. Reaction (500 μ L) rates were measured in a Shimadzu spectrophotometer by monitoring the increase in absorbance at 290 nm due to formation of PR-ATP ($\epsilon_{290\text{nm}} = 3,600 \text{ M}^{-1} \text{ cm}^{-1}$)¹⁷ in 1-cm path length quartz cuvettes (Hellma). A typical reaction mixture contained 3 mM ATP, 2 mM PRPP, 1 μ M *PaHisG_S* in the presence or absence of 32 μ M *PaHisZ*. Inhibition was tested by running the reaction in the presence of 1 mM histidine. Reactions were started by addition of PRPP. Control reactions lacked either ATP, PRPP, *PaHisG_S*, or *PaHisZ*. Background rates in the absence of *PaHisZ* were subtracted from *PaATPPRT* rates. All kinetic measurements were performed in duplicates.

Measurement of PaATPPRT equilibrium dissociation constant (K_D). Initial velocities were measured in the presence of 3 mM ATP, 2 mM PRPP, 1 μ M *PaHisG_S*, and varying concentrations of *PaHisZ* (2 – 32 μ M), at different temperatures (283 – 308 K). Reaction mixtures were incubated at each temperature for 5 min prior to addition of PRPP to start the reaction. *PaHisZ-PaHisG_S* K_D values were obtained by fitting initial rate data to a kinetic equation (*vide infra*).

PaATPPRT and PaHisG_S substrate saturation curves. *PaATPPRT* initial rates were measured at various temperatures (283 – 308 K) in the presence of 1.1 μ M *PaHisG_S*, 20 μ M *PaHisZ*, saturating concentrations of one substrate, and varying concentrations of the other, either ATP (0.4 – 5.6 mM) or PRPP (0.1 – 2.0 mM, 283 – 293 K; 0.2 – 4.0 mM, 298 – 308 K). Initial rates for *PaHisG_S* at various temperatures (283 – 313 K) were determined in the presence of 2.2 μ M *PaHisG_S*, saturating concentrations of one substrate, and varying concentrations of

the other, either ATP (0.2 – 2.8 mM, 283 – 298 K; 0.4 – 5.6 mM, 303 – 313 K) or PRPP (0.1 – 2.0 mM, 283 – 298 K; 0.2 – 4.0 mM, 303 – 313 K). Reaction mixtures were incubated at each temperature for 5 min prior to addition of PRPP to start the reaction.

Comparison of PaHisGs and PaATPPRT reactions by ^{31}P -NMR spectroscopy. Reactions (500 μL) contained 100 mM tricine pH 8.5, 15 mM MgCl_2 , 100 mM KCl, 4 mM DTT, and 20 μM MtPPase, 3 mM ATP, 3 mM PRPP, and either 44 μM PaHisGs (for PaHisGs reactions) or 22 μM PaHisGs and 50 μM PaHisZ (for PaATPPRT reactions). Control reactions contained all but PaHisGs. Individual standards contained either 3 mM ATP, 3 mM PRPP, or 3 mM phosphate in 100 mM tricine pH 8.5, 15 mM MgCl_2 , 100 mM KCl, 4 mM DTT, and 20 μM MtPPase. All reactions were run in duplicates. Samples were incubated for 40 min at 293 K, after which 100 μL of D_2O was added to each sample, and proteins were removed by passage through 10,000 MWCO Vivaspin centrifugal concentrators. ^1H -decoupled ^{31}P -NMR spectra were recorded on a Bruker Avance 500 (200 MHz ^{31}P) spectrometer using water as solvent and D_2O to achieve solvent lock. A total of 128 scans were collected and averaged for each sample, with 3 s of relaxation time. Data were processed in MestReNova. Chemical shifts (δ) are given in parts per million (ppm) with coupling constants (J values) reported to the nearest 0.1 Hz.

CD spectroscopy of PaHisGs and PaHisZ. Both proteins storage buffer was exchanged to a solution of 10 mM K_2HPO_4 pH 8.0, 10 mM KF by successive passages through 10,000 MWCO Vivaspin centrifugal concentrators. CD spectra were acquired at 293 K in a Biologic MOS-500 spectrometer outfitted with a Peltier temperature controller in a 1-cm path length cuvette containing 0.5 mg mL^{-1} of either PaHisGs or PaHisZ. Blank spectra were recorded with the same buffer in the absence of protein and subtracted from the protein spectra. Protein Thermal denaturation was assessed by monitoring CD absorbance at either 208 nm for

PaHisG_S or 222 nm for *PaHisZ* at varying temperatures (283 – 343 K with 2.5 K increments). All measurements for CD spectra and thermal denaturation were collected in duplicates.

Kinetic and thermal denaturation data analysis. Kinetic data were analysed by the nonlinear regression function of SigmaPlot 13 (SPSS Inc.). Data points and error bars in graphs are represented as mean $\pm$ standard error, and kinetic and equilibrium constants are presented as mean $\pm$ fitting error. Initial rate data with varying concentrations of *PaHisZ* were fitted to eq 1. The concentration of *PaATPPRT* at any concentrations of *PaHisG_S* and *PaHisZ* was calculated according to eq 2. Substrate saturation data were fitted to eq 3. Data for temperature-dependence of the rates were fitted to eqs 4 and 5. In eqs 1 – 5, v is the initial rate, $V_{\max}$ is the maximal velocity, G is the concentration of *PaHisG_S*, Z is the concentration of *PaHisZ*, K_D is the equilibrium dissociation constant, *PaATPPRT* is the concentration of *PaHisG_S*–*PaHisZ* complex, S is the concentration of the varying substrate, k_{cat} is the steady-state turnover number, K_M is the Michaelis constant, E_T is total enzyme concentration, T is the temperature in Kelvin, R is the gas constant, E_a is the energy of activation, and A the Arrhenius pre-exponential factor.

$$v = V_{\max} \frac{(G+Z+K_D) - \sqrt{(G+Z+K_D)^2 - 4 G Z}}{2 G} \quad \text{eq 1}$$

$$PaATPPRT = \frac{(G+Z+K_D) - \sqrt{(G+Z+K_D)^2 - 4 G Z}}{2} \quad \text{eq 2}$$

$$\frac{v}{E_T} = \frac{k_{\text{cat}} S}{K_M + S} \quad \text{eq 3}$$

$$k_{\text{cat}} = A e^{\frac{-E_a}{R T}} \quad \text{eq 4}$$

$$\ln k_{\text{cat}} = \frac{-E_a}{R} \left(\frac{1}{T} \right) + \ln A \quad \text{eq 5}$$

Thermal denaturation data were fitted to eq 6. Here, F_U is fraction unfolded, T is the temperature in K, T_m is the melting temperature, m_T is the slope of the transition region, m_U

and b_U are slope and intercept, respectively, of the unfolded baseline, and m_F and b_F , those of the folded baseline.⁴⁷

$$F_U = \frac{(m_U T - b_U) - (m_F T - b_F)}{1 + \left(\frac{T}{T_m}\right)^{m_T}} + m_F T - b_F \quad \text{eq 6}$$

RESULTS AND DISCUSSION

Purification of PaATPPRT. *PaHisG_S* and *PaHisZ* were purified to homogeneity via Ni-affinity chromatography. No other bands were detected by Coomassie Blue-stained SDS-PAGE (Figure S1). Typical yields were 8 mg and 10 mg of protein per gram of cells for *PaHisG_S* and *PaHisZ*, respectively. As a result of His-tag removal with TEVP, both proteins have an additional residue, Gly, at their N-termini. ESI-MS analyses confirmed the molecular mass of each protein as 25,215 for *PaHisG_S* and 43,082 for *PaHisZ* (Figure S2), closely matching their theoretical values of 25,213 and 43,085.

Crystal structure of PaATPPRT. The three-dimensional structure of *PaATPPRT* holoenzyme was solved and refined to 2.34-Å resolution, and refinement statistics are summarised in Table 1. Atomic coordinates were deposited in the Protein Data Bank under PDB ID 5M8H. As published with *TmATPPRT*¹² and *LlATPPRT*,¹¹ *PaATPPRT* is a heterooctamer consisting of four *PaHisZ* subunits in the centre flanked on each side by two *PaHisG_S* subunits (Figure 1A and B). *PaATPPRT* crystals, in the P2₁ space group, had one heterooctamer per asymmetric unit. Even though ATP and histidine were present in the crystallisation mixture, no clear electron density was observed for either ligand. No electron density was observed for glutaraldehyde either, which suggests that the proteins may not have been cross-linked in the crystal.

PaHisG_S subunits. *PaHisG_S* subunits overlay with slightly different root-mean-square deviation (rmsd) values depending on the subunit. For example, rmsd's of 0.316, 0.177, and

0.384 Å result from overlaying subunit E with F, G, and H, respectively, over 208 C α atoms. *PaHisG_S* displays two domains, domain 1 and domain 2 (Figure 1C). The former includes residues 20 – 115 and 197 – 228, while the latter consists of residues 116 – 196. Residues 1 – 19 are disordered and were excluded from the model. Domain 1 has six β -strands split into two small antiparallel β -sheets, β 2- β 3 and β 4- β 9- β 5, with β 1 forming a three-stranded parallel sheet with β 3 and β 4. These β -sheets are encircled by five α -helices, α 1, α 2, α 3, α 7, and α 8. Domain 2 includes a β -strand core arranged in a three-stranded parallel β -sheet (β 7- β 6- β 8), and a three-stranded antiparallel β -sheet (β 8- β 5- β 9). This β -strand core is sandwiched between α 4 on one side and α 5 and α 6 on the other. This domain arrangement is similar to those of *TmHisG_S*¹² and *LtHisG_S*,¹¹ and to the catalytic domains of *HisG_L*.^{8, 19}

Each pair of *PaHisG_S* makes up a homodimer in which subunits interact in a head-to-tail arrangement (Figure 1B). An interface score of 1 was calculated for this interaction, suggesting that it is responsible for homodimer formation. Scores of 0 indicate no interaction, while scores of 1 indicate that the interaction leads to complex formation.⁴⁶ The interface covers approximately 1,200 Å², representing 10.6% of the solvent accessible area. This encompasses thirty-one residues, mostly from α 2, β 2, β 3, β 7, and α 5. Based on the crystal structure of PRPP-bound *LtATPPR*, the active site is expected to lie on a crevice between domains 1 and 2.²¹ The absence of electron density for ATP along with the absence of PRPP from the crystallisation mixture preclude a precise mapping of the *PaATPPRT* active-site interactions with substrates. Nevertheless, amino acid sequence alignment with *HisG_S* orthologues using Clustal Omega shows that all PRPP-interacting residues and two ATP-interacting ones are conserved in *PaHisG_S* (Figure S3).

PaHisZ subunits. Each subunit has a large N-terminal domain (NTD), encompassing residues 1 – 318, and a small C-terminal domain (CTD), residues 324 – 387, connected by a

loop (Figure 1D). NTD is comprised of a six-stranded antiparallel β -sheet, $\beta 5$ - $\beta 6$ - $\beta 11$ - $\beta 10$ - $\beta 9$ - $\beta 7$, flanked by two small β -strands, $\beta 1$ and $\beta 8$, which run parallel to $\beta 5$ and $\beta 7$, respectively. This β -sheet seats inside a bowl-like arrangement of nine α -helices ($\alpha 1$, $\alpha 4$ - $\alpha 9$, $\alpha 11$, $\alpha 12$) and two 3_{10} -helices ($\omega 10$ and $\omega 13$). Helix $\alpha 4$ connects NTD with a small subdomain consisting of a three-stranded antiparallel β -sheet, $\beta 2$ - $\beta 4$, and two small α -helices, $\alpha 2$ and $\alpha 3$. The NTD is structurally similar to *TmHisZ*¹² and *LHisZ*.¹¹ CTD consists of a three-stranded parallel β -sheet, $\beta 12$ - $\beta 14$, with $\beta 15$ antiparallel to $\beta 14$, and two α -helices, $\alpha 14$ and $\alpha 15$. This CTD is absent in *TmHisZ*¹² and *LHisZ*,¹¹ but present in *HisRS*.¹² *HisZ* and *HisRS* are predicted to have diverged from a common ancestor.⁹ *PaHisZ* appears to have retained that CTD while *TmHisZ* and *LHisZ* have lost it.

Each pair of *PaHisZ* constitutes a homodimer in which subunits interact in a head-to-tail arrangement (Figure 1A). A relatively low interface score of 0.23 was calculated for this interaction, in spite of involvement of both NTD and CTD. The interface covers approximately 3,300 Å², representing 15.6% of the solvent accessible area. Interacting residues are found mainly in the regions 1-134 and 352-369. *PaHisZ* subunits overlay with slightly different rmsd values depending on the subunit. For example, rmsd's of 0.798, 0.922, and 0.131 Å result from overlaying subunit A with B, C, and D, respectively. When the four *PaHisZ* chains are superimposed, the regions showing the largest differences involve $\alpha 7$ - $\alpha 9$, $\omega 10$, and $\alpha 11$ in the NTD as well as $\alpha 14$, $\alpha 15$, $\beta 14$, and $\beta 15$ in the CTD (Figure S4). These regions are at opposing ends of the monomer and do not interact with other regions in the complex.

PaHisGs-PaHisZ interface. The interaction between catalytic and regulatory subunits is shown in Figure 1E. The contact area is not as extensive as those of the homodimers. The interface between *PaHisZ* NTD and *PaHisGs* domain 1 covers approximately 860 Å², while that between *PaHisZ* NTD and *PaHisGs* domain 2 (of neighbouring subunit), 320 Å².

Calculated interface scores of only 0.03 and 0.01 for domains 1 and 2, respectively, suggests a relatively weak interaction between the two subunits. The interaction is stabilised by eight hydrogen bonds and three salt bridges for NTD-domain 1 and by four hydrogen bonds for NTD-domain 2. In addition, several hydrophobic residues (Leu107, Leu108, Leu202, Val204, Val207, Leu216, Leu220) contributed by *PaHisGs* are buried in this region.

Cold-adaptations in PaATPPRT. Psychrophilic enzymes are suggested to be more flexible than their mesophilic counterparts as an adaptation to achieve high catalytic rates at low temperature, and this flexibility is purported to be more accentuated in the active site.^{23, 27} Conversely, a compelling hypothesis recently proposed that cold-adaptation is achieved by increased flexibility at outer regions of enzymes, at the protein-solvent interface, while the core is significantly more rigid. In molecular dynamics simulations of cold-adapted trypsin, this variation in flexibility was assessed according to the magnitudes of thermal motion factors (B-factors).⁴⁸ The distribution of high and low B-factor regions throughout the catalytic and regulatory subunits in *PaATPPRT* (Figure 2A) is more complex. *PaHisZ* has a relatively “cold” core surrounded by “hot” outer regions (Figure 2B). However, the catalytic subunit is dominated by high B-factors with only small portions of intermediate flexibility (Figure 2C), even though the outer regions seem to be the “hottest” ones. Interestingly, residues expected to form the PRPP binding site are found in a relatively “hot” region of *PaHisGs*, while residues expected to interact with ATP are located on a comparatively more rigid region.

PaATPPRT K_D values. Enzymatic activity by *PaATPPRT* is dependent on the concentration of *PaHisZ* (Figure 3A), even though the regulatory subunit has no catalytic activity. This indicates that *PaHisZ* binds to *PaHisGs* to form the *PaATPPRT* holoenzyme, activating *PaHisGs* catalysis, as expected.^{11, 12} Fitting the data to eq 1 provided *K_D* values over a range of temperatures (Table S1). The general trend suggests a decrease in affinity between *PaHisZ* and *PaHisGs* as the temperature increases (Figure 3B). Nevertheless, the total variation

in K_D is small, with a minimum value of $4.7 \pm 0.8 \mu\text{M}$ at 283 K and a maximum of $9.5 \pm 1.2 \mu\text{M}$ at 303 K. Knowledge of K_D allows calculation, using eq 2, of $PaATPPRT$ concentrations for any given concentrations of $PaHisZ$ and $PaHisGs$, a requirement for determination of k_{cat} in subsequent kinetic analyses.

Temperature-dependence of PaATPPRT kinetics. Psychrophilic enzymes are subjected to an intricate balance between protein stability and catalytic activity to ensure function at low temperature.^{23, 24, 27, 28} To investigate how temperature variation influences catalysis by $PaATPPRT$, steady-state kinetic constants were determined at several temperatures, and their values are summarized in Table S2. $PaATPPRT$ followed Michaelis-Menten kinetics for both ATP (Figure 3C) and PRPP (Figure 3D). Values of k_{cat} are 3-fold smaller and 3-fold larger, respectively, than those reported for $LlATPPRT$ ²¹ and for the homo-hexameric *M. tuberculosis* enzyme.³ $PaATPPRT$ K_M for PRPP is 14-fold, 46-fold, 63-fold, and 33-fold higher in comparison with those determined at similar temperatures for mesophilic ATPPRT's from *M. tuberculosis*, *C. jejuni*, *S. typhimurium*, and *L. lactis*, respectively.^{3, 4, 14, 21} $PaATPPRT$ K_M for ATP is 6-fold, 17-fold, and 2-fold, higher in comparison with those determined at similar temperatures for mesophilic ATPPRT's from *M. tuberculosis*, *C. jejuni*, and *S. typhimurium*, respectively.^{3, 4, 14} The exception is $LlATPPRT$ K_M for ATP, which is 1.5-fold lower than that for $PaATPPRT$.²¹ High K_M is a common feature of cold-adapted enzymes.²⁴

$PaATPPRT$ k_{cat} increases with temperature between 283 – 303 K (Figure 3E), and data fitting to eq 4 resulted in an energy of activation (E_a) of $7.6 \pm 0.5 \text{ kcal mol}^{-1}$. An E_a of $7.9 \pm 0.6 \text{ kcal mol}^{-1}$ was obtained by fitting the data to eq 5 in the more traditional Arrhenius plot (Figure 3F). However, Arrhenius behaviour is sharply interrupted at 308 K (excluded from the fit) as the k_{cat} decreases to a value similar to that found at 298 K (Figure 3E). Decrease in k_{cat} at relatively mild temperatures is characteristic of psychrophilic enzymes, likely a consequence of portions of the protein beginning to unfold^{23, 24} (*vide infra*).

PaHisG_S catalysis in the absence of PaHisZ. *PaHisG_S* displays catalytic activity without activation by *PaHisZ* (Figure 4). Moreover, while 1 mM histidine almost eliminates *PaATPPRT*-catalysed reaction, it has no effect on *PaHisG_S* reaction (Figure 4). The latter observation also eliminates the possibility that enzymatic activity in the absence of *PaHisZ* might be due to some undetectable amount of contaminating *E. coli* *ATPPRT* (*EcATPPRT*), consisting of His_{GL}, in *PaHisG_S* preparations, since just 200 μ M histidine would be enough to inhibit 95% of *EcATPPRT*.¹⁵ A similar observation of independent catalysis has been recently reported for *LHisG_S*.²²

In order to gather evidence in addition to the increase in UV absorbance at 290 nm^{1, 17} that *PaHisG_S* is catalysing the same reaction as *PaATPPRT*, both reactions were compared by ³¹P-NMR spectroscopy (Figure 5). Spectra of both reactions, control, ATP, PRPP, and inorganic phosphate, along with assignment of chemical shifts, are shown in Figure S5. The spectra of reactions catalysed by *PaHisG_S* (Figure 5A) and by *PaATPPRT* (Figure 5B) are essentially identical, and both differ from the control reaction which lacked *PaHisG_S* (Figure 5C). The most conspicuous difference, besides the increase in the inorganic phosphate peak at 2.29 ppm, is the appearance of a peak at 3.30 ppm in the *PaHisG_S* (Figure 5A, inset) and *PaATPPRT* (Figure 5B, inset) reactions, which is absent in the control (Figure 5C, inset). This peak corresponds to the phosphorus in the N¹-5-phospho- β -D-ribose moiety of PR-ATP, while the same phosphorus has a chemical shift of 3.49 ppm in PRPP (Figure S5B). These observations confirm the products of *PaHisG_S*-catalysed reaction are the same as those of the reaction catalysed by *PaATPPRT*. Combined, the results in this section qualitatively support those recently published for *LHisG_S*,²² and indicate that the prevailing assumption that HisG_S is catalytically inactive in the absence of HisZ^{11, 12} should be revised.

Temperature-dependence of PaHisG_S kinetics. To explore the effect of temperature on *PaHisG_S* catalytic rate, steady-state kinetic constants were determined at several temperatures,

and their values are summarized in Table S3. As observed with *Pa*ATPPRT, *Pa*HisG_S follows Michaelis-Menten kinetics for both ATP (Figure 6A) and PRPP (Figure 6B). Comparison between k_{cat} values at corresponding temperatures for *Pa*ATPPRT and *Pa*HisG_S demonstrates that *Pa*HisZ only modestly activates *Pa*HisG_S catalysis, as the holoenzyme k_{cat} values are larger than those for *Pa*HisG_S by 4-fold at the lowest temperatures to just 2-fold at the highest ones. This is significantly different than what is found with *Ll*ATPPRT, in which *Ll*HisZ confers 10-fold activation as compared with *Ll*HisG_S alone.²² Interestingly, *Pa*HisG_S K_M values for both substrates are smaller in comparison with *Pa*ATPPRT, most markedly at the lowest temperatures which are likely to be closer to those experienced by *P. arcticus* in its natural environment.³⁴ It is possible to hypothesise that the primary function of *Pa*HisZ is to serve as a means of feedback inhibition by histidine, not to activate *Pa*HisG_S.

*Pa*HisG_S k_{cat} increases with temperature between 283 – 308 K (Figure 6C), and data fitting to eq 4 resulted in an E_a of $10.0 \pm 0.3 \text{ kcal mol}^{-1}$, whereas an E_a of $10.5 \pm 0.3 \text{ kcal mol}^{-1}$ resulted from fitting the data to eq 5 in the Arrhenius plot (Figure 6D). Surprisingly, Arrhenius behaviour is obeyed up to 308 K, with *Pa*HisG_S departing from it only at 313 K (excluded from the fit) (Figure 6C). This points to *Pa*HisZ rendering *Pa*HisG_S catalytic activity more thermolabile since *Pa*ATPPRT abandons Arrhenius behaviour by 308 K (Figure 3F).

Thermal denaturation of PaHisG_S and PaHisZ. To interrogate the possibility that *Pa*HisZ tertiary structure is more thermolabile than that of *Pa*HisG_S, thermal denaturation of both proteins was assessed following their CD signal. Both *Pa*HisG_S and *Pa*HisZ yielded CD spectra typical of folded proteins (Figure 6E).⁴⁹ Thermal denaturation data were fitted to eq 6 (Figure 6F) and yielded melting temperature (T_m) values of $316 \pm 1 \text{ K}$ for *Pa*HisG_S and $312 \pm 2 \text{ K}$ for *Pa*HisZ. In spite of the modest difference in T_m 's, the data are in qualitative correlation with the Arrhenius behaviour of *Pa*HisG_S and *Pa*ATPPRT k_{cat} 's. It should be pointed out that the T_m 's might be affected if the proteins were in complex with each other. Unfortunately, all

attempts to measure the T_m for *PaATPPRT* led to protein precipitation as the temperature approached 313 K at the high protein concentrations needed to ensure that most of *PaHisZ* and *PaHisG_S* were in complex.

Two hypotheses could be invoked to explain the departure from Arrhenius behaviour by *PaATPPRT*. The simpler one would be that free *PaHisZ* (in molar excess to *PaHisG_S*) starts to unfold at 308 K, which leads to dissociation of bound *PaHisZ* from *PaATPPRT* as the binding equilibrium is displaced. *PaHisG_S* would then be the main contributor to k_{cat} at 308 K, explaining the lower value. Nonetheless, this hypothesis is in disagreement with the *PaATPPRT* K_D value at 308 K. The K_D 's at 303 K and 308 K are the same within experimental error (Table S1). Hence there is no loss in affinity between the two proteins at 308 K to account for the decrease in *PaATPPRT* k_{cat} .

Another hypothesis is that *PaATPPRT* is mostly folded at 308 K, but there is a temperature-induced loosening of the interactions at *PaHisG_S-PaHisZ* interfaces. This would not be enough to cause dissociation of the complex, as reflected by the unchanged K_D at 308 K. However, this loosening would be sufficient to interfere with activation of *PaHisG_S* catalytic activity by the regulatory subunit, explaining the reduction in k_{cat} .

Utilising cold-adapted enzymes in biocatalysis reduces costs and emissions as heating is not required.³⁰ As synthetic biology efforts towards histidine production in microbes depend on maintaining efficient catalysis while eliminating histidine feedback inhibition of ATPPRT,⁶ *PaHisG_S* provides an advantageous system to be explored.

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SUPPORTING INFORMATION AVAILABLE

Figures S1 – S5, and Tables S1 – S3. This material is available free of charge via the internet at <http://pubs.acs.org>.

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Table 1. Crystallographic data and refinement statistics

Parameter	Value
PDB ID	5M8H
Wavelength (Å)	1.5418
Resolution (Å) ^a	30.63 - 2.34 (2.38-2.34)
Total No. reflections	390,223
No. unique reflections	110,587
Multiplicity	3.5 (2.4)
Completeness (%)	97.6 (81.0)
<I/sigI>	6.3 (0.9)
R_{merge} ^b	0.135 (0.969)
CC(1/2)	0.994 (0.366)
Wilson B (Å ²)	34.1
Space group	P2 ₁
Unit cell parameters	
<i>a</i> (Å)	94.02
<i>b</i> (Å)	146.73
<i>c</i> (Å)	101.90
α (°)	90.00
β (°)	102.4
γ (°)	90.00
Refinement	
No. reflections (working set)	105092

No. reflections (R_{free} test set)	5467
$R_{\text{work}}/R_{\text{free}}^{c,d}$	0.23/0.27
rmsd bond distances (Å)	0.010
rmsd bond angles (°)	1.438
No molecules per a.u.	4 each of HisG and HisZ
Protein atoms	18229
Ligands	37
Water molecules	629
Average B factor (Å ²)	
Protein	50.14
Ligands	57.76
Waters	35.75
Ramachandran plot	
Favoured (%)	97.4
Allowed (%)	2.6
Outliers	0

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^aValues in parentheses are for the highest resolution shell.

^b $R_{\text{merge}} = \sum |I_{(hkl)} - I| \times 100 / \sum |I_{(hkl)}|$, where the average intensity I is taken over all symmetry equivalent measurements and $I_{(hkl)}$ is the measured intensity for a given observation.

^c $R_{\text{work}} = \sum_{hkl} ||F_o(hkl)| - |F_c(hkl)|| / \sum_{hkl} |F_o(hkl)|$.

^d $R_{\text{free}} = R_{\text{factor}}$ for a test set of reflections (5%).

Scheme 1. ATP phosphoribosyltransferase-catalysed synthesis of N^1 -(5-phospho- β -D-ribose)-ATP, the first step of histidine biosynthesis.

FIGURE LEGENDS

Figure 1. Crystal structure of *PaATPPRT*. (A) Ribbon diagram of the hetero-octamer with four *PaHisGs* subunits (red and orange) and four *PaHisZ* subunits (green, blue, cyan, and yellow). (B) Top view of image shown in (A). Images in A and B created in PyMol. (C) Ribbon diagram of a *PaHisGs* monomer, with α -helices in green and β -strands in blue. (D) Ribbon diagram of a *PaHisZ* monomer, with α -helices in green, β -strands in blue, and 3_{10} -helices in beige. (E) *PaHisZ* NTD (ribbon diagram in green) and *PaHisGs* domain 1 (ribbon diagram in orange) and *PaHisGs* domain 2 of neighbouring subunit (ribbon diagram in red) interfaces. Side chains of residues participating in inter-subunit interactions are shown as stick models, with nitrogen in blue, oxygen in red, and carbon in either green (*PaHisZ*), orange (*PaHisGs* domain 1), and red (*PaHisGs* domain 2). Images generated in CCP4MG.

Figure 2. B-factor distribution in (A) the *PaATPPRT* hetero-octamer, (B) *PaHisZ* monomer, and (C) *PaHisGs* monomer. Images generated in PyMol.

Figure 3. Temperature studies of *PaATPPRT*. (A) Rate-dependence of *PaATPPRT* reaction on *PaHisZ* concentration at 283 K (pink), 288 K (red), 293 K (cyan), 298 K (blue), 303 K (green), and 308 K (grey). Lines represent data fitting to eq 1. (B) Temperature variation of *PaHisGs-PaHisZ* K_D . (C) and (D) *PaATPPRT* saturation curves for ATP and PRPP, respectively, at 283 K (pink), 288 K (red), 293 K (cyan), 298 K (blue), 303 K (green), and 308 K (grey). Lines represent data fitting to eq 3. (E) Deviation of *PaATPPRT* k_{cat} from Arrhenius behaviour at 308 K. Line represents data fitting to eq 4, excluding data point at 308 K. (F) Linear Arrhenius plot of *PaATPPRT* k_{cat} , excluding the data point at 308 K. Line represents data fitting to eq 5.

Figure 4. *PaHisGs* catalysis in the absence of *PaHisZ*. (A) Time course of product formation for control without *PaHisGs* (red), control without PRPP (green), reaction with *PaATPPRT* at

1 μ M (blue), *Pa*ATPPRT reaction in the presence of 1 mM histidine (yellow), reaction with *Pa*HisGs at 1.1 μ M (cyan) and 2.2 μ M (pink) without *Pa*HisZ, and reaction with *Pa*HisGs at 2.2 μ M in the presence of 1 mM histidine without *Pa*HisZ (grey). (B) Bar graph representation of the rates. Colour scheme the same as in A.

Figure 5. ^{31}P -NMR spectra of (A) *Pa*HisGs reaction, (B) *Pa*ATPPRT reaction, and (C) control in the absence of *Pa*HisGs. Insets in A and B are close-ups of the spectra between 4.0 and 3.0 ppm showing the peak at 3.30 ppm, corresponding to the phosphorus in the N^1 -5-phospho- β -D-ribose moiety of PR-ATP, which is missing in inset in C.

Figure 6. Temperature studies of *Pa*HisGs and *Pa*HisZ. (A) and (B) *Pa*HisGs saturation curves for ATP and PRPP, respectively, at 283 K (pink), 288 K (red), 293 K (cyan), 298 K (blue), 303 K (green), 308 K (grey), and 313 K (black). Lines represent data fitting to eq 3. (C) Deviation of *Pa*HisGs k_{cat} from Arrhenius behaviour at 313 K. Line represents data fitting to eq 4, excluding data point at 313 K. (D) Linear Arrhenius plot of *Pa*HisGs k_{cat} , excluding the data point at 313 K. Line represents data fitting to eq 5. (E) CD spectra of *Pa*HisGs (blue) and *Pa*HisZ (pink). (F) Thermal denaturation curves for *Pa*HisGs (blue) and *Pa*HisZ (pink). Lines represent data fitting to eq 6.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

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Kinetics and structure of a cold-adapted hetero-octameric ATP phosphoribosyltransferase[†]

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