**Supplementary material**

**Results**

**MJ1130 is an iron metalloprotein**

The purified MJ1130 protein exhibits a pink color in solution resulting from a broad absorbance band centered at 492 nm as observed for the *Pyrococcus abyssi* ortholog PaKae1 (Hecker, et al., 2007) (Figure S1A). This broad absorbance corresponds to a charge-transfer transition from a tyrosinate to a Fe(III) ion suggesting that MJ1130 contains a Fe$^{3+}$ ion. This was confirmed by the EPR (electron paramagnetic resonance spectroscopy) spectrum obtained at pH 8.0 (Figure S1B) which exhibits features characteristic of a high-spin (S=5/2) Fe(III) monomer. Compared to the spectrum recorded on PaKae1 (Hecker, et al., 2007), the features are sharper and the low field signals have a smaller amplitude relative to the sharp g=4 signal. The broad signals (g=8.2 and 5.4) for PaKae1 had been interpreted as a mixture of species or at least conformational heterogeneity at the level of the iron site. In the case of MJ1130, the site seems to be more homogeneous and it exhibits lower rhombicity. Thus, MJ1130 also contains a Fe$^{3+}$ ion and the sharper signal indicates a lower conformational heterogeneity of the site compared to that of PaKae1.

**The autophosphorylation activity of MjBud32 is inhibited by Mj-Kae1**

We tested the kinase activity of the archaeal MJ1130 protein and of the MjKae1 and MjBud32 subdomains that we expressed independently in *E. coli*. The fusion protein, as well as the Bud32 moiety did not exhibit kinase activity on casein in our experimental conditions. However, both the fusion protein and the two subdomains exhibited an autophosphorylation activity (previously observed in the case of PaKae1, Hecker et al., 2007). These autophosphorylation activities were stronger when the proteins were tested separately (Figure S5). Furthermore, the autophosphorylation of MjBud32 was completely suppressed by the addition of the Kae1 moiety. Taken together our results indicate that archaeal Bud32 phosphotransferase activity is inhibited by Kae1. This fits well with the fact that we could not detect AMPPNP in the nucleotide binding site of MjBud32, suggesting that the presence of Kae1 maintains Bud32 in an inactive conformation both in the crystal and in solution.
Material and methods

Amplification and cloning

Wild-type and mutant constructs of archaeal Mj1130, MjKae1 and MjBud32
The sequence encoding archaeal Mj1130, MjKae1 and MjBud32 (Kae1 and Bud32 domains of MJ1130 respectively) proteins were amplified by polymerase chain reaction using genomic DNA of Methanocaldococcus jannaschii strain DSM 2661 as template and specific primers containing a NdeI or a six histidine-tag fused to a NotI site as forward and reverse specific primers respectively. The PCR products were cloned into pGEM-T easy vector (Promega), then digested by appropriate restriction enzymes and finally subcloned in a modified pET-9a expression vector (Novagen) between Ndel and NotI restriction sites.

Wild-type and mutant constructs of yeast kae1 and bud32-his
Yeast Kae1p and Bud32p-his were coexpressed in E. coli in a polycistronic vector (namely 6-pK-Sc-Ka,Bu-a), derived from pET28 vector (Novagen). The kae1 gene was amplified by PCR and ligated at the NotI restriction site followed by the Sc-bud32-his gene which has a 6His-tag encoding sequence at the 3’ end. E. coli expression with 6-pK-Sc-Ka,Bu-a leads to single transcripts bearing the two genes that both have their own rbs (ribosome binding site). Mutants of either kae1 or bud32 in the polycistron have been constructed with the QuikChange mutagenesis kit (Stratagene) by using 6-pK-Sc-Ka,Bu-a plasmid as template and specific oligonucleotides (see Table S1) containing mutation according to the supplier's instructions.

Expression and purification of recombinant proteins

Expression and purification of archaeal Mj1130, MjKae1 and MjBud32 proteins
Expression of recombinant proteins were performed at 37°C using a transformed E. coli Rosetta (DE3) pLysS by the appropriate recombinant plasmid in LB medium containing kanamycin (50 µg/ml) and chloramphenicol (34 µg/ml). When the cell culture reached an OD600 of 0.7, expression of recombinant protein was induced by 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 hours. Cells were harvested by centrifugation,
resuspended in a Tris-HCl 20 mM pH 8.0, NaCl 200 mM (or 500 mM in the case of MjBud32), β-mercaptoethanol 5 mM buffer and stored overnight at -20°C. Cell lysis was completed by sonication. Cell extract was then centrifuged at 10,000 g for 15 min. at 4°C to remove cellular debris and aggregated proteins. His-tagged protein from soluble fraction was purified by gravity-flow chromatography on a nickel nitriloacetate agarose resin (Ni-NTA) column (Qiagen Inc.) according to the manufacturer’s recommendations followed by a gel filtration step on a Superdex™ 75 column (GE Healthcare). The homogeneity of the protein was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determinate by measuring the absorbance at 280 nm using specific molar absorption coefficient.

**Co-expression and co-purification of yeast Kae1p and Bud32p-his**

Kae1p and Bud32p-his have been expressed by transforming *E. coli* Rosetta pLysS (Novagen) with 6-pK-Sc-Ka,Bu-a plasmid. Bacteria were grown at 37°C in 2xYT medium supplemented with kanamycin and chloramphenicol until the OD reached 0.6 to 0.8 and the growth temperature was then shifted to 15°C for one hour. Expression was then triggered by adding 0.5 mM of IPTG and the culture was left overnight at 15°C. Bacteria were harvested and stored at -20°C after being resuspended in Tris-HCl 20 mM pH 7.5, NaCl 200 mM, 2-mercaptoethanol 5 mM (lysis buffer). The same expression protocol has been used for the mutated proteins.

Co-purification of either wt or mutated Kae1p and Bud32p-his has been performed by IMAC (Immobilized Metal Affinity Chromatography). The bacteria were lyzed on ice by sonication and centrifuged at 20000 g for 25 min. The supernatant was recovered, precipitated with 0.2 % (final concentration) of polymin P, incubated on ice for 15 min. and centrifuged at 20000 g for 25 min. The resulting supernatant was mixed with Protino NiIDA resin (Macherey Nagel) for 30 min at 4°C and loaded onto a column. The resin was then washed with lysis buffer and the bound proteins were successively eluted with 3 fractions containing the same buffer supplemented with 100 mM, 200 mM and 400 mM imidazole. The two pellets obtained after the two centrifugation steps were also pooled in the same volume of lysis buffer used for the resuspension of bacteria and kept for subsequent analyses. All the fractions obtained after each purification step were analysed by SDS-PAGE or Western blotting.
Expression and purification of the yeast proteins used for the kinase assay

Bud32p-his, Bud32p-his D161A and Grx4p-his have been expressed in *E. coli* BL21 Gold (Stratagene) and purified by using the same pET20-derivative plasmids and same protocols as described by Lopreiato *et al.* (2004). Kae1p and Bud32p-his have been co-expressed and co-purified as already described above (see “Co-expression and co-purification of yeast Kae1p and Bud32p-his”) except that we added a size exclusion chromatography step on Superdex™ 200 (GE Healthcare) just after elution from IMAC in order to isolate an equimolar ratio of Kae1p and Bud32p-his in the binary complex.

Phosphotransferase assay on archaeal proteins

The phosphotransferase activity of kinase was assayed on archaeal MJ1130, MjKae1 and MjBud32 proteins. MJ1130, MjBud32, MjKae1 (approx. 5 µg) were incubated with 25 µM [\(\gamma^{32}P\)]ATP (1 µCi/reaction, specific activity of 6000 Ci.mmol\(^{-1}\)) in 20 µl of buffer containing 50 mM Hepes-HCl pH 7.5, 100 mM NaCl, 1 mM DTT, 10 mM MnCl\(_2\) for 30 min. at 72°C. After incubation, reactions were stopped by the addition of loading buffer and the samples analyzed by electrophoresis (SDS-PAGE). The gels were then soaked in 16% TCA for 10 min. at 90°C and neutralized in 100 mM phosphate buffer pH 7.4. This procedure eliminates most unincorporated \([32P]\), non-protein material, phosphorylation on His and Asp residues and enriches samples for O-phosphorylated proteins (Lévine *et al.*, 2006). The treated gels were finally stained in neutralization buffer supplemented with 5% TCA and 0.1% Coomassie blue and radiolabelled proteins visualized by autoradiography.

Optical and spectroscopic characterization

Visible spectra were recorded on a Cary 50 spectrophotometer (Varian Inc.) using 200 µg or 100 µg of recombinant MJ1130 or PaKae1 diluted in Tris-HCl 20 mM pH 8.0, NaCl 200 mM. EPR spectra were realized in the same conditions at 10 K on a Bruker Elexsys E500. Prior to measurements, the tubes were degassed with helium gas to remove oxygen from the samples.
**Co-Immunoprecipitation**

Extracts were prepared from wild type yeast cells, expressing an HA-tagged version of chromosomal Bud32p and ectopically expressing wt or mutant Kae1TAP from a pCM185 plasmid (the endogenous untagged version of Kae1p was not deleted in these strains). Yeast cells were first grown in selective minimal medium to minimize plasmid loss and transferred to 200 mL of YPDA medium for roughly two generations (OD600 1.0). Collected cells were broken in 1:1 w/v of AGK Buffer (Hepes 20 mM pH 7.9, MgCl2 1.5 mM, KCl 200 mM, Glycerol 10%, DTT 0.5 mM, AEBSF 1 mM, Complete antiprotease cocktail, Boehringer), extracts were cleared by two centrifugation steps (14000 rpm for 45 min and 54000 rpm for 30 min) and dialyzed against Buffer D (Hepes 20 mM pH 7.9, KCl 50 mM, EDTA 0.2 mM, Glycerol 20%, DTT 0.5 mM). Protein amounts have been determined by Bradford assay. TAP-tagged Kae1 proteins have been immunoprecipitated by incubating (3 hrs at 4°C) 3 mg of whole cell extracts with Sepharose IgG-beads affinity matrix (Amersham). Beads were washed 3 times with IPP150 Buffer (Tris-HCl 10 mM pH 8; NaCl 150 mM; NP40 0.1%) and bound proteins were eluted with 1X Laemmli for SDS-PAGE and Western blot analysis. Filters were probed with anti-HA antibody to reveal the presence of Bud32-HA in Kae1-TAP immunoprecipitates. Where indicated immunoprecipitations and washings were performed at different salt concentrations (50, 250, 500 mM NaCl).

**Western Blotting**

Rabbit polyclonal antibodies (Kae1p) against Kae1p have been raised by Genecust Company after providing them with Kae1p-his protein purified in denaturing condition. After SDS-PAGE, proteins were transferred onto a Hybond-P membrane (GE Healthcare) in Tris-HCl 25 mM pH 8.5, Glycin 192 mM, methanol 20%. The membrane was probed with α-Kae1p (dilution 1:20000) for one hour in TBST (Tris-HCl 10 mM pH 8, NaCl 150 mM, Tween20 0.2%) then washed 3 times (10 minutes) in TBST and probed with the secondary anti-rabbit antibody (GE Healthcare) for one hour in TBST. After 3 wash steps the membrane was finally labelled with the ECL western blotting kit (GE Healthcare) according to the supplier’s instructions.
References


Legends to figures

Figure S1: Spectroscopic analysis of MjKae1/Bud32

A. Light absorbance spectra of native MjKae1/Bud32 (a) and PaKae1 (b, Hecker et al. 2007). Spectra were obtained using 200 µg MjKae1/Bud32 or 100 µg PaKae1 in Tris-HCl 20 mM pH 8.0, NaCl 200 mM buffer.

B. Electron paramagnetic resonance spectra of MjKae1/Bud32 (a) and PaKae1 (b, Hecker et al., 2007). The corresponding g-values are indicated for some features. Experimental conditions: microwave power 0.5 mW (top) and 0.1 mW (bottom); microwave frequency 9.38 GHz; modulation amplitude 1 mT; modulation frequency 100 kHz; temperature 10 K. Some g values are indicated on the EPR spectra.

Figure S2: Sequence alignment of MjKae1 orthologs.

Strictly conserved residues are in white on a black background. Partially conserved amino acids are boxed. Stars below the alignment indicate residues involved in the interface.

Figure S3: Sequence alignment of MjBud32 orthologs.

Strictly conserved residues are in white on a black background. Partially conserved amino acids are boxed. Filled circles below the alignment indicate residues involved in the interface. The domains of the protein kinases and functionally relevant signatures are indicated below the alignments.

Figure S4. Superposition of the ATP binding site from MjKae1 and PaKae1.

Stereo view representation of the ATP binding site from MjKae1, colored in blue. Upperposed PaKae1 is colored in yellow with the AMPPNP ligand shown as ball and sticks and the iron ion depicted as a yellow sphere. The 2Fo-Fc electron density map of MJ1130 is shown in blue around the AMPPNP molecule.

Figure S5. Autophosphorylation of MjBud32 is inhibited by Mj-Kae1.

MJ1130, MjBud32 and/or MjKae1 proteins (approx. 5 µg) were subjected to phosphorylation for 30 min. at 72°C in 20 µl of a buffer containing 50 mM Hapes-HCl pH 7.5, 500 mM NaCl, 10 mM MnCl₂ and 25 µM [γ-32P]ATP. Radiolabelled proteins were separated by SDS-PAGE. Gels were then soaked in 16% TCA for 10 min. at 90°C to eliminate radiolabelled
nucleotides, stained by Coomassie blue (lower panel) and finally analysed by PhosphorImager (upper panel). 1. Mj1130 alone, 2. MjBud32 (a) incubated with MjKae1 (b), 3. MjKae1 alone, 4. MjBud32 alone.

Figure S6. MjKae1/Bud32 structure.
A. Superposition of the two MjKae1/Bud32 protomers present in the crystal asymmetric unit. For clarity, only MjBud32 is shown entirely.
B. Ribbon representation of MjKae1/Bud32 monomer with the loops containing insertions in yeast homologues coloured in red (N-terminal part) and green (C-terminal part).
C. Molecular surface representation of sequence conservation of Kae1 and Bud32 orthologs. Colouring is from light grey (poorly conserved) to red (highly conserved). AMPPNP is shown as sticks.
Table S1. Primers used for mutant constructs. Mutagenic codons are underlined.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bud32p-R72E</td>
<td>5'-GGCACAACAAAAACATGAAGATGATGCG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GGGCTCTCATTTCAGATGCG-3'</td>
</tr>
<tr>
<td>Kae1p-D236R</td>
<td>5'-CGATCAAGGGGATCGTCATCTATTGAGTGG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CCACCTGTGGAAGAAGCCATCCCCCTTACTG-3'</td>
</tr>
<tr>
<td>Kae1p-E292R</td>
<td>5'-CGGCGATCGTATAACAGAAGAGC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GCTCTTTCTGTAATACGCATGGCG-3'</td>
</tr>
<tr>
<td>Kae1p-E292,295R</td>
<td>5'-CTATCCAGGCATATGAGGCTAGTACAAAGGCTATGGCTACGTT-3'</td>
</tr>
<tr>
<td></td>
<td>5'-AACGTGATGCACTGTTATACGAAAATGCGAATAG-3'</td>
</tr>
</tbody>
</table>

Table S2. *Saccharomyces cerevisiae* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303</td>
<td>ura3-1, ade2-1, his3-11, trp1-1, leu2-3,112, CAN1-100</td>
<td></td>
</tr>
<tr>
<td>DLY344</td>
<td>as W303, MATa, Δkae1::HIS, pFL38-KAE1 (URA3)</td>
<td>this study</td>
</tr>
<tr>
<td>DLY764</td>
<td>as W303, MATa, BUD32-HA::HIS</td>
<td>this study</td>
</tr>
<tr>
<td>DLY785</td>
<td>as W303, MATalpha, Δbud32::KAN, pFL38-BUD32 (URA3)</td>
<td>this study</td>
</tr>
<tr>
<td>DLY1251</td>
<td>as W303, MATalpha, Δkae1::HIS, pCM185-KAE1 (TRP1)</td>
<td>this study</td>
</tr>
<tr>
<td>DLY1252</td>
<td>as W303, MATalpha, Δkae1::HIS, pCM185-kae1D236R (TRP1)</td>
<td>this study</td>
</tr>
<tr>
<td>DLY1253</td>
<td>as W303, MATalpha, Δkae1::HIS, pCM185-kae1E292R (TRP1)</td>
<td>this study</td>
</tr>
<tr>
<td>DLY1254</td>
<td>as W303, MATalpha, Δkae1::HIS, pCM185-kae1E295R (TRP1)</td>
<td>this study</td>
</tr>
<tr>
<td>DLY1255</td>
<td>as W303, MATalpha, Δkae1::HIS, pCM185-kae1E292-295RR (TRP1)</td>
<td>this study</td>
</tr>
<tr>
<td>DLY1256</td>
<td>as W303, MATalpha, Δbud32::KAN, pCM185-BUD32 (TRP1)</td>
<td>this study</td>
</tr>
<tr>
<td>DLY1261</td>
<td>as W303, MATalpha, Δbud32::KAN, pCM185-bud32K57R60EE (TRP1)</td>
<td>this study</td>
</tr>
<tr>
<td>DLY1262</td>
<td>as W303, MATalpha, Δbud32::KAN, pCM185-bud32R72E (TRP1)</td>
<td>this study</td>
</tr>
<tr>
<td>DLY1263</td>
<td>as W303, MATalpha, Δbud32::KAN, pCM185-bud32R204E (TRP1)</td>
<td>this study</td>
</tr>
</tbody>
</table>
Figure S1

A.

![Absorbance vs Wavelength](image)

B.

![dY''/dB vs Magnetic Field](image)
Figure S4
Figure S6