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Archaeal aIF2B interacts with eukaryotic translation initiation factors eIF2 α and eIF2B α : implications for aIF2B function and eIF2B regulation

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Abstract

Translation initiation is down-regulated in eukaryotes by phosphorylation of the α subunit of eIF2, which inhibits its guanine nucleotide exchange factor eIF2B. The N-terminal S1 domain of phosphorylated eIF2 α interacts with a subcomplex of eIF2B formed by the three regulatory subunits, α /GCN3, β /GCD7, and δ /GCD2, blocking the GDP-GTP exchange activity of the catalytic ϵ -subunit of eIF2B. These regulatory subunits have related sequences and also have sequences in common with many archaeal proteins, some of which are involved in methionine salvage and CO₂ fixation. Our sequence analyses predicted however that members of one phylogenetically distinct and coherent group of these archaeal proteins (designated aIF2Bs) are functional homologues of the α , β and δ subunits of eIF2B. Three of these proteins, from different Archaea, have been shown to bind *in vitro* to the α subunit of the archaeal aIF2 from the cognate Archaeon. In one case, the aIF2B protein was shown further to bind to the S1 domain of the α subunit of yeast eIF2 *in vitro* and to interact with eIF2B α /GCN3 *in vivo* in yeast. The aIF2B-eIF2 α interaction was however independent of eIF2 α phosphorylation. Mass spectrometry has identified several proteins that copurify with aIF2B from *Thermococcus kodakaraensis* and these include aIF2 α , a sugar-phosphate nucleotidyltransferase with sequence similarity to eIF2B ϵ , and several large subunit (50S) ribosomal proteins. Based on this evidence that aIF2B has functions in common with eIF2B, the crystal structure established for an aIF2B was used to construct a model of the eIF2B regulatory subcomplex. In this model, the evolutionarily conserved regions and sites of regulatory mutations in the three eIF2B subunits in yeast are juxtaposed in one continuous binding surface for phosphorylated eIF2 α .

Keywords

aIF2B; eIF2; eIF2B; Archaea; *S. cerevisiae*; *T. kodakaraensis*; translation initiation; phosphorylation; GCN3; GCD2; GCD7; GCD6; ribosomes

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Introduction

Protein synthesis in eukaryotic cells is regulated by phosphorylation of serine 51 (Ser51) of the α -subunit of translation initiation factor 2. This converts the GDP-bound form of eIF2 into a competitive inhibitor of eIF2B, the guanine nucleotide exchange factor (GEF) of eIF2. Only eIF2-GTP can form a ternary complex (TC) with methionyl initiator tRNA (Met-tRNA_i) and assemble the preinitiation complex on the small (40S) ribosomal subunit, and so eIF2 phosphorylation reduces the rate of general translation initiation. This inhibition of eIF2B occurs during stress or starvation conditions when eIF2 α is phosphorylated by the activation of eIF2 α -specific protein kinases, reprogramming the cell's translational profile ^{1; 2}.

The eIF2B is comprised of five non-identical subunits in 1:1:1:1:1 stoichiometry ³, four of which are essential proteins in yeast ⁴. Only the C-terminal segment of the largest (ϵ) subunit (GCD6 in yeast) is required for GEF activity in vitro, but this has a lower specific activity than the complete eIF2B complex ⁵. Three of the subunits (α /GCN3, β /GCD7, and δ /GCD2) not only support substrate binding/catalysis by the ϵ subunit but also play regulatory roles as mediators of the inhibition of GEF activity by eIF2 phosphorylated on its α -subunit (eIF2 [α P]). These eIF2B regulatory subunits have related sequences and form a stable subcomplex that binds the α subunit of eIF2 in vitro, dependent on Ser51 phosphorylation ^{6; 7; 8; 9}. Mutations in the regulatory subunits or in eIF2 α that weaken this interaction abrogate the inhibition of eIF2B activity by phosphorylated eIF2 in vivo ^{9; 10}, arguing that tight binding of the phosphorylated α -subunit of eIF2 (eIF2 α -P) to the regulatory subcomplex of eIF2B is necessary to inhibit GEF activity. We have proposed that phosphorylation of eIF2 α stabilizes eIF2 binding to eIF2B in a manner that prevents a productive interaction of the catalytic ϵ -subunit with the GDP binding pocket in eIF2 γ ⁶. The GEF activity of eIF2B that lacks the α -subunit is resistant to inhibition by eIF2(α P) ^{6; 11; 12}, and consistent with this, deletion of the α /GCN3 subunit has no deleterious effects on yeast except under starvation conditions when eIF2B inhibition by eIF2(α P) is essential for survival ⁴.

Much of the information processing machinery found in eukaryotes appears to have originated in the archaea ¹³, or the common ancestor to archaea and eukaryotes ¹⁴, whereas the eukaryotic operational genes are most closely related to bacterial genes ¹⁵. There are structural and functional homologues of eIF2 in Archaea, designated aIF2, that in the GTP-bound state bind Met-tRNA_i and transfer it to the small ribosomal subunit ^{4; 16; 17; 18; 19}. However, Ser51 is not conserved in archaeal aIF2 α , and although *Pyrococcus horikoshii* aIF2 α was phosphorylated in vitro on Ser48 by the eukaryotic eIF2 α kinase PKR, the significance of this has not been determined ²⁰. Archaeal genomes do not encode recognizable homologues of the catalytic ϵ -subunit of eIF2B, and aIF2 β lacks a region corresponding to the N-terminal portion of eIF2 β that binds to the catalytic segment of eIF2B ϵ and stimulates nucleotide exchange ^{5; 21}. Given these observations, and that aIF2 from *Sulfolobus solfataricus* binds GDP and GTP with equal affinity ¹⁶, it seems that there is no GEF for aIF2 nor a mechanism that regulates GDP-GTP exchange on aIF2 in Archaea.

Archaeal genomes do, however, encode three families of proteins with sequences related to the eIF2B regulatory subunits. A member of one of these families has been documented to be a ribose-1,5-biphosphate isomerase (RBPI) and to participate in CO₂ fixation ²². Based on sequence and motif conservation, the second family of proteins is likely to comprise the methylthioribose-1-phosphate isomerases (MTNAs), which function in methionine salvage ^{22; 23}. Our detailed sequence analyses leads us to conclude that the third family is most closely related to the eIF2B regulatory subunits, and the experiments reported here were therefore undertaken to determine if members of this family (designated aIF2Bs) have functions in common with the eIF2B regulatory subunits. We have established that aIF2Bs from several species do bind to the α -subunits of their cognate aIF2s, and that one such aIF2B binds to yeast

eIF2 α but does so independently of Ser51 phosphorylation. When isolated directly from *Thermococcus kodakaraensis* aIF2B co-purified with aIF2 α , with a protein that has sequences in common with eIF2B ϵ , and with several ribosomal large-subunit proteins. With this support for aIF2B family members interacting with aIF2 and participating in translation initiation, we used the crystal structure established for aIF2B from *Pyrococcus horikoshii* to construct a model of the regulatory subcomplex of yeast eIF2B. The model predicts that the three regulatory subunits assemble to generate a complex that has one composite binding surface to which eIF2 α -P could bind and so regulate eIF2B activity.

Results

Alignments of eIF2B-related sequences identify putative archaeal eIF2B homologs

To identify archaeal proteins likely to be homologs of the eIF2B regulatory subunits, rather than MTNA or RBPI enzymes, we conducted BLAST searches using the eIF2B-related protein from *Pyrococcus horikoshii* PH0440, as the query sequence and constructed multiple sequence alignments of the 90 proteins with the highest BLAST scores. Bacterial and eukaryotic MTNAs have 8 highly conserved sequence motifs, designated *mI* to *mVIII* with 6 invariant residues (underlined in Fig. 1) that likely make contacts with the bound phosphate in the active site, or function as catalytic residues^{23; 24}. Of the 47 archaeal genomes analyzed, ~90% encode one protein highly related to established MTNAs. These proteins contain all the conserved motifs, and almost all of the invariant residues, and so are very likely to be functional archaeal MTNAs (MTNA-like sequences in Fig. 1 & Table 1). Approximately half of the genomes that encode such a MTNA sequence also encode a protein that has sequences related to motifs *mII*, *mIII* and *mV-VIII* but that is truncated at the N-terminus and so lacks *mI* and the N-terminal β -sheet component of MTNAs²³. These proteins do retain the invariant Cys in *mIV* but have 8 residues deleted (residues GxxATxxx) from this motif. They include the RBPI encoded in *T. kodakaraensis* TK0185, and it seems likely therefore that these proteins are all RBPIs or proteins with related enzyme activities (RBPI-like sequences in Fig. 1 & Table 1).

Approximately 50% of the archaeal genomes encode a protein that lacks the N-terminal β -sheet of MTNAs and has highly degenerate versions of all the motifs except for, in some cases, *mVI* and *mVIII*. These proteins lack both the conserved Cys in *mIV* and the 8 residues deleted from this motif in the RBPI family. Given that eIF2B regulatory subunits also lack most or all of the motifs conserved in MTNAs²³, this third family of archaeal proteins were the most likely candidates for aIF2Bs (aIF2B-like sequences in Fig. 1 & Table 1) and this included PH0440, the protein that had already been crystallized and designated aIF2B α ²⁵.

Phylogenetic analyses of the 90 archaeal sequences together with 7 putative MTNAs and 24 eIF2B regulatory subunits from plant, fungal or animal species confirmed, with high bootstrap values, that these proteins formed three phylogenetically coherent and discrete groups (Fig. 2). A dendrogram with similar topology was generated when the trees were rooted to the RBPI sequences (Fig. 2) or midpoint-rooted (not shown). All of the archaeal proteins designated as MTNA-like in Table 1 formed one monophyletic clade that also contained all 7 putative eukaryotic MTNAs (Fig. 2), although some of these proteins are annotated in UniProt as members of the eIF2B family. All of the putative archaeal RBPIs formed a second group that did not contain any eukaryotic sequences, and all the putative archaeal aIF2Bs formed a third monophyletic clade which also contained all 24 eIF2B sequences. Thus, the aIF2B sequences are more closely related to the eukaryotic eIF2B subunits than to eukaryotic or archaeal MTNAs or archaeal RBPI sequences.

It is interesting that in the *Euryarchaea* aIF2B is encoded in all *Thermococcales*, *Thermoplasmatales* and *Halobacteriales* genomes but not in the genomes of class I or class II methanogens (Table 1). *Haloarcula marismortui* is unique in having three different aIF2Bs.

The single aIF2B present in *P. horikoshii* forms homodimers²⁵, but with three aIF2Bs present in *H. marismortui* the opportunity exists to form heterodimers, or even a heterotrimer as formed by three eIF2B regulatory subunits in eukaryotes.

***T. acidophilum* aIF2B binds tightly to cognate aIF2 α and the S1 domain of yeast eIF2 α**

To determine if the aIF2B proteins were functionally analogous to the eukaryotic eIF2B regulatory subunits, we generated and purified recombinant aIF2B and aIF2 α , tagged at their N-termini with hexahistidine (His₆) or GST, respectively, from three archaeal species (*P. horikoshii*, *P. furiosus* and *T. acidophilum*). These proteins were assayed for their ability to form complexes by GST pull-down assays. Western blot analyses revealed that all three His₆-tagged aIF2B proteins (His-aIF2B) interacted significantly with the GST-aIF2 α from the same species, but did not bind to GST alone (Fig. 3A). The amount of His-aIF2B binding to GST-aIF2 α was consistently greatest for the proteins from *T. acidophilum* and, based on Coomassie Blue staining, a molar excess of His-aIF2B bound to GST-aIF2 α from this species, suggesting the formation of a stable complex between oligomeric His-aIF2B and GST-aIF2 α (Fig. 3B). Complex formation by the two proteins from *P. horikoshii* could be enhanced, however, by addition of 200 mM NaCl to the binding reaction (Fig. 3C, lanes 4-11).

The same procedure was used to determine if His₆-tagged aIF2B from *T. acidophilum* could also form a stable complex with recombinant yeast eIF2 α /SUI2 fused to GST. Remarkably, His₆-aIF2B bound to GST-eIF2 α (Fig. 4B, lanes 1-4 vs. 17-20), and deletions from the C-terminus revealed that this interaction required only the ~140 N-terminal residues of eIF2 α in the GST-eIF2 α fusion (lanes 5-16). The subcomplex formed by the eIF2B regulatory subunits similarly binds to this region of eIF2 α 9' 10, which includes the β -barrel "S1 domain" (residues 1-87) and the Ser51 phosphorylation target 26 (Fig. 4A). When the N-terminal 39 or 44 amino acid residues were removed from the eIF2 α in the GST-eIF2 α fusion, eliminating β -strands 1 and 2, or 1, 2 and 3, respectively (Fig. 4A), binding by His-aIF2B was substantially reduced (Fig. 4C) consistent with aIF2B binding requiring an intact β -barrel in the S1 domain of eIF2 α .

We asked next whether the yeast eIF2B regulatory subcomplex resembles aIF2B in requiring an intact S1 domain in eIF2 α for complex formation, as this was not fully established in our previous studies. To this end, we examined the effects of removing β 1- β 2 from the GST-eIF2 α fusion on binding of the eIF2B regulatory subcomplex present in whole cell extracts (WCEs) of a yeast strain over-expressing all three regulatory subunits (α /GCN3, β /GCD7, δ /GCD2). Increased binding of the over-expressed subunits was observed previously in extracts of a strain overproducing all three subunits, but not in strains overproducing only a single subunit, thus establishing that a trimeric regulatory subcomplex was responsible for the increased binding to GST-eIF2 α . Furthermore, prior phosphorylation of GST-eIF2 α on Ser51 by the human eIF2 α kinase PKR was required for this binding reaction⁹. As expected, we observed binding of α /GCN3, β /GCD7, and δ /GCD2 in the WCEs to GST-eIF2 α only when the latter was phosphorylated by PKR in vitro (Fig. 4D, lanes 6-9). Also as seen previously, the C-terminally truncated fusion GST-eIF2 α ₁₋₁₄₀ was not phosphorylated by PKR and did not bind to the regulatory subunits (lanes 14-17).

PKR binds to the N-terminal region of eIF2 α , making direct contact with residues in the β -barrel²⁷, but it additionally requires the adjacent helical domain of eIF2 α (residues 88-182) for efficient phosphorylation of Ser51^{10; 27} (Fig. 4A). This latter requirement can account for our finding that the C-terminally truncated fusion GST-eIF2 α ₁₋₁₄₀ was not phosphorylated by PKR. Unexpectedly, we found that the fusion truncated from the N-terminus and lacking β 1- β 2, GST-eIF2 α ₄₀₋₃₀₅, was still phosphorylated by PKR under these conditions, presumably owing to the high substrate concentrations employed in the reactions (Fig. 4D, lanes 10-13). Nevertheless, the phosphorylated protein did not bind the eIF2B regulatory subcomplex. These

last results suggest that an intact β -barrel in the eIF2 α S1 domain is required for tight binding to the eIF2B regulatory subcomplex, just as concluded above for aIF2B.

Our finding that the eIF2B regulatory subcomplex cannot bind to the C-terminally truncated fusion GST-eIF2 α_{1-140} (Fig. 4D) is ostensibly at odds with the fact that His-aIF2B binds efficiently to this fusion (Fig. 4B). However, the eIF2B regulatory subcomplex binds only to phosphorylated GST-eIF2 α , whereas His-aIF2B can bind the unphosphorylated protein. Hence, failure of the eIF2B regulatory subcomplex to interact with GST-eIF2 α_{1-140} can be explained simply by its inability to be phosphorylated, without the need to propose that eIF2 α_{1-140} lacks a critical determinant for binding eIF2B that would be dispensable for binding aIF2B.

Although aIF2B bound unphosphorylated GST-eIF2 α , we investigated whether this interaction was stimulated by Ser51 phosphorylation, given that phosphorylation of GST-eIF2 α by PKR is required for its tight binding to the eIF2B regulatory subcomplex (Fig. 5A, lanes 1-8). However, we found that His-aIF2B bound equally well to un-phosphorylated and phosphorylated GST-eIF2 α (lanes 9-16), consistent with phosphorylation of Ser51 not increasing the affinity of aIF2B for eIF2 α .

Some substitutions in the S1 domain of eIF2 α reduce binding of phosphorylated GST-eIF2 α to the eIF2B regulatory subcomplex *in vitro*^{9; 10} and confer a Gcn⁻ (general control noninducible) phenotype in yeast by impairing translational induction of the *GCN4* gene. Phosphorylation of eIF2 α by the kinase GCN2 in amino acid starved cells, with attendant reduction in eIF2-GTP formation, reduces general protein synthesis but specifically stimulates translation of *GCN4* mRNA owing to its unique reinitiation mechanism. As GCN4 is a transcriptional activator of amino acid biosynthetic enzymes subject to general amino acid control, its induction enables increased amino acid production (reviewed in 28). Gcn⁻ substitutions in eIF2 α that weaken binding of eIF2 α -P to the eIF2B regulatory subcomplex eliminate the inhibition of eIF2B by eIF2(α P) and thereby block induction of *GCN4* and amino acid biosynthetic genes under its control. Using the GST pull-down assay, we determined if the substitutions in two such representative Gcn⁻ variants of eIF2 α (R88T and L84F) affected His₆-aIF2B binding. However, these substitutions did not reduce recombinant His₆-aIF2B binding to GST-eIF2 α (Fig. 5B) and, therefore, the aIF2B interaction with eIF2 α is both independent of Ser51 phosphorylation and insensitive to substitutions that weaken binding of phosphorylated eIF2 α to the eIF2B regulatory subcomplex in yeast.

aIF2B binds eIF2B α in vivo and destabilizes the eIF2B-eIF2 complex

Having found that *T. acidophilum* aIF2B binds tightly to GST-eIF2 α *in vitro* we asked if it would bind to eIF2, or interact with eIF2B regulatory subunits, in yeast cells. We expressed GST-aIF2B or GST alone from the galactose-inducible *GAL1* promoter, precipitated the fusion protein from WCEs with glutathione-agarose beads, and probed the co-precipitated proteins for eIF2B and eIF2 subunits. Although eIF2 was not detectable in the bound fraction, the eIF2B subunit α /GCN3 was co-precipitated with GST-aIF2B, but not with GST alone (Fig. 6A). These findings suggest that aIF2B forms a stable complex with α /GCN3 in yeast, possibly a heterodimer resembling the PH0440 homodimer²⁵.

Consistent with previous findings^{7; 29}, when aliquots of WCE from cells expressing GST alone were incubated with antibodies against eIF2B subunit ϵ /GCD6, the immunoprecipitated complexes contained all five eIF2B subunits and eIF2 α (Fig. 6B, lanes 1-2). However, the amounts of the three eIF2B regulatory subunits and eIF2 α that coimmunoprecipitated with ϵ /GCD6 from WCE from cells over-expressing GST-aIF2B were greatly reduced as compared with extracts containing GST alone, whereas the amount of co-precipitating γ /GCD1 was not influenced by GST-aIF2B over-expression (Fig. 6B, lanes 1-4). One explanation would be that

GST-aIF2B forms a heterodimer with α /GCN3, leading to dissociation of α /GCN3 from eIF2B and attendant loss of the other two regulatory subunits and eIF2 from the eIF2B-eIF2 complex. This would leave only γ /GCD1 tightly bound to ϵ /GCD6, ie. the two subunits of the eIF2B catalytic subcomplex⁶. Consistent with this possibility, deleting *GCN3* from the parental strain yields coimmunoprecipitation results almost indistinguishable from those obtained by over-expression of GST-aIF2B in yeast (Fig. 6B, lanes 3-6).

A *gcn3Δ* mutation has no detrimental effects on cell growth in nutrient rich media, in which translational control of *GCN4* is dispensable³⁰, and this is also the case for over-expression of GST-aIF2B (data not shown). Apparently, neither *gcn3Δ* nor GST-aIF2B over-expression substantially impairs the essential functions of eIF2B in translation initiation *in vivo*. Hence, as discussed further below, the disruption of eIF2-eIF2B complexes observed in these situations most likely occurs only *in vitro* during the stringent washes applied to the complexes immobilized on protein A-Sepharose with ϵ /GCD6 antibodies or on glutathione-Sepharose by GST-aIF2B.

aIF2B interacts with aIF2 α , a sugar-phosphate nucleotidyl transferase related to eIF2B ϵ , and to 50S ribosomal proteins in *T. kodakaraensis*

To determine if aIF2B interacts with aIF2 or other components of the translational machinery in archaeal cells, we replaced the gene encoding aIF2B in *T. kodakaraensis* (*TK1047*) with a modified allele tagged at the C-terminus with HA and His₁₀ epitopes. As documented in Fig. 2, the encoded amino acid sequence of *TK1047* is very similar to that of *P. horikoshii* aIF2B (PH0440). Cultures of *T. kodakaraensis* H4 and H2 expressing tagged and untagged versions of aIF2B, respectively, were grown as described³¹, and WCEs prepared and subjected to Ni²⁺-chelation chromatography. Column fractions from the H4 extract that contained HA-His₁₀-aIF2B were identified by Western blot analyses and pooled. Fractions eluting under the same conditions from the H2 WCE were also collected and pooled. Mass spectrometry using multidimensional protein identification technology (MudPit) was used to identify proteins that co-purified with the HA-His₁₀-aIF2B from *T. kodakaraensis* H4 using the search engine Mascot (www.matrixscience.com). Table 2 lists the proteins with Mascot scores higher than 100 for which two or more independent peptides were identified in the H4 sample (at a 5% false discovery rate) and that were not identified in the H2 sample. In addition to a large number of peptides matching aIF2B itself, the H4 samples contained two peptides that match the α subunit of aIF2 and peptides matching 9 large (50S) ribosomal subunit proteins, but no small (30S) subunit proteins. High Mascot scores were also recorded for the β and γ subunits of aIF2 (293 and 515, respectively) reflecting identification of 4-6 unique peptides for these proteins in the H4 samples. These sequences are not included in Table 2, however, as subsets of β and γ peptides were also present in the H2 samples, albeit with lower Mascot scores (191 and 246 for aIF2 β and aIF2 γ , respectively). It appears that these two aIF2 subunits bind non-specifically to the Ni²⁺ resin.

To substantiate the conclusion that aIF2 α interacts with aIF2B, we purified HA-His₁₀-aIF2B and limited the mass spectrometry analysis to co-purified material in gel slices that contained polypeptides with the predicted mobility of aIF2 α (31.6 kD). Again, there were two different peptides matching the aIF2 α sequence in the gel slice from the H4 strain (Mascot score 219) but none in the corresponding gel slice from the H2 strain. This identification of two peptides in a narrow molecular mass fraction greatly increases the likelihood that they derive from aIF2 α . Given the presence of aIF2 α and multiple 50S subunit proteins exclusively in H4 fractions, and the greater sequence coverage of aIF2 β and aIF2 γ peptides in the H4 versus H2 fractions, it seems very likely that aIF2B interacts with aIF2 and 50S subunits in *T. kodakaraensis* cells.

It is intriguing that aIF2B also interacted specifically with a sugar-phosphate nucleotidyl transferase (TK0955; Table 2) that is predicted to synthesize GDP-mannose from mannose-1-phosphate and GTP (KEGG orthology database entry K00966 at <http://www.genome.jp/kegg/ko.html>). Both subunits of the eIF2B catalytic subcomplex, ϵ /GCD6 and γ /GCD1, resemble sugar-phosphate nucleotidyl transferases, harboring both nucleotidyl transferase (NUCT) and isoleucine-rich hexapeptide repeat (I-patch) domains³². The TK0955 sequence contains regions that conform to the consensus sequences for both NUCT and I-patch domains, and can be aligned with both domains in ϵ /GCD6 (Fig. 6C and data not shown). It is possible that aIF2B interacts with TK0955 in *T. kokakarensis* in a manner similar to the interaction of the regulatory subunits with catalytic subunits in the eIF2B holoprotein.

Structural modeling of eIF2B regulatory subunits and mutations

Taken together, the biochemical results described above confirmed that the aIF2B proteins we studied exhibited three distinct interactions that argue for functional conservation with eIF2B regulatory subunits. Namely, they form specific associations with aIF2 α and eIF2 α proteins, with a regulatory subunit of eIF2B (α /GCN3), and with a protein containing the NUCT and I-patch domains present in eIF2B catalytic subunits. Given this conservation, we took advantage of the crystal structure of *P. horikoshii* aIF2B to generate 3-D models of the yeast eIF2B regulatory subunits and predict the locations of Gcn⁻ regulatory mutations.

The crystal structure of the antiparallel homodimer of PH0440²⁵ is shown in a ribbons depiction in Fig. 7A using blue and magenta or red and yellow, for the α -helical and β -stands, respectively, of the two protomers. In the view shown in panel I (left-hand side of Fig. 7A), the same face of each protomer in the homodimer is visible, rotated 180° relative to the other protomer. The view in panel II of Fig. 7A is rotated 180° from that of panel I to show the opposite face of the homodimer.

To construct a model of the β /GCD7 regulatory subunit, we first aligned PH0440 with the sequences of eIF2B β from different plants, animals and fungi. PH0440 can be aligned with eIF2B β sequences throughout their lengths except for four inserts of variable length in eIF2B β , designated *i-1* to *i-4* (Fig. S1). We then projected the degree of sequence conservation at each residue (excluding the inserts) onto a surface representation of the PH0440 homodimer to produce a model of a hypothetical β /GCD7 homodimer that displays the predicted surface-conserved residues in the eukaryotic proteins (Fig. 7B, panels I & II). Interestingly, most of the highly conserved residues (in magenta) lay on the face of β /GCD7 visible in panel I, whereas the opposite face in panel II is enriched in nonconserved residues.

We then asked whether the more highly conserved face of the β /GCD7 model is enriched in residues that were implicated in the inhibition of eIF2B by eIF2(α P) by the isolation of Gcn⁻ substitutions at these positions that abrogate this regulation. We first identified residues in PH0440 that align with 15 spontaneous Gcn⁻ mutations isolated previously in *GCD7*^{7; 33} and projected these residues on the 3-D model of β /GCD7. Eight such mutations alter predicted surface-exposed residues, of which 6 map to the highly conserved face of β /GCD7. The PH0440 residues altered by these *GCD7* mutations (D178Y, R254C, P291S, V292A, I348V and N357I) are colored in dark blue and labeled on the homodimer shown on the right-hand panel of Fig. 8A, and also indicated by stars on the left-hand panel to reveal their sequence conservation among β /GCD7 homologs. (For convenience, we labeled some substitutions on one protomer and some on the other protomer.) The remaining two mutations alter surface residues visible when the dimer is rotated by 90° (F82L, Fig. 8B) or on the nonconserved face of the model (K329E, Fig. 8C).

Six of the seven remaining Gcn⁻ mutations in *GCD7* alter residues located in one of the four inserts, which do not align with PH0440 residues. However, all three inserts containing these mutations are predicted to project from the conserved face of the protein, as shown by the residues colored in black that immediately flank the inserts in the right-hand panels of Fig. 8A-B. Because the Y305C, P306L, and S359G mutations in this group alter amino acids within 3 residues of the beginning of insert-3 or -4 (Fig. 8), they likely alter residues exposed on the conserved face of β /*GCD7*. Because the L117S, I118T and S119P Gcn⁻ mutations in this group affect residues roughly in the middle of *i-1* we cannot predict their locations. The final Gcn⁻ mutation (G218R) in β /*GCD7* alters a residue buried below the surface of α IF2B (not shown).

In summary, 9 of 12 Gcn⁻ mutations whose locations can be predicted alter surface-exposed residues on the conserved face of the β /*GCD7* model. We showed previously that one of the Gcn⁻ mutations mapping on the conserved face (D178Y, Fig. 8A), decreases α IF2 α -P binding to the α IF2B regulatory subcomplex in vitro⁹. This suggests that binding determinants for α IF2 α -P reside on the conserved face of β /*GCD7*. It is significant that D178Y and 6 other Gcn⁻ mutations on the conserved face span ~50% of the protein length but define a much smaller proportion of the total surface area in the predicted 3-D structure. Based on the proximity of these 7 residues in the folded protein, we propose that they define a contiguous surface in β /*GCD7* that is critical for tight binding of α IF2(α P) to the α IF2B regulatory subcomplex.

Turning next to the δ /*GCD2* subunit, the C-terminal ~400 residues of δ /*GCD2* from different eukaryotic species can be aligned with α IF2B except for seven inserts and an extension at the C-terminus (Supplementary Fig. S2). Remarkably, most of the highly conserved residues in δ /*GCD2* project to the same face of the molecule that contains the majority of highly conserved residues in β /*GCD7*, whereas the opposite face is enriched with non-conserved residues (except for the dimer interface) (Fig. 7C). Importantly, 9 of the 10 Gcn⁻ mutations isolated in δ /*GCD2*⁷ are also predicted to alter surface-exposed residues on the conserved face (dark blue residues and stars in Fig. 9A, right and left panels, respectively), and the tenth (E377K) alters a residue lacking in α IF2B that corresponds to insert *i-5* on the conserved face (black residues in Fig. 9A, right).

Strikingly similar findings were obtained for the α /*GCN3* subunit of α IF2B, with the most highly conserved residues identified by sequence alignment (Fig. S3) projected on the same face of the model that harbors most of the conserved residues in the δ /*GCD2* and β /*GCD7* models (Fig. 7D). Moreover, all seven Gcn⁻ mutations we isolated in α /*GCN3*⁷ alter surface-exposed residues (5 blue residues in Fig. 9C), or residues in small inserts only a few positions removed from a surface exposed residue on the conserved face (F73L and F240L/I; black residues in Fig. 9C).

Previously, we isolated α /*GCN3* mutations with a distinct phenotype, known as Gcd⁻ (general control derepressed), in which *GCN4* translation is constitutively induced in the absence of α IF2 α phosphorylation by *GCN2*³⁴. A subset of these *gcn3^c* mutations appear to mimic the inhibitory effect of α IF2 α phosphorylation on α IF2B by increasing the binding of unphosphorylated α IF2 α to the α IF2B regulatory subunits, as their Gcd⁻ phenotypes are suppressed by Gcn⁻ mutations in α IF2 α that also suppress the effects of α IF2 α phosphorylation on *GCN4* expression and cell growth³³. If these *gcn3^c* mutations increase binding of the α IF2B regulatory subcomplex to unphosphorylated α IF2 α , as we have proposed, then some of them should map to the conserved face of α /*GCN3* in proximity to Gcn⁻ mutations that confer resistance to α IF2(α P). In fact, this prediction was met for 6 of the 7 *gcn3^c* mutations that alter surface-exposed residues in the α /*GCN3* model (orange residues in Fig. 9C), and also for the D71N *gcn3^c* mutation that maps to insert *i-1* on the conserved face (black residues). Our findings are consistent with the idea that Gcn⁻ and Gcd⁻ mutations affecting the conserved face

of α /GCN3 delineate its contribution to the binding surface for eIF2(α P) in the eIF2B regulatory subcomplex. Whereas Gcn⁻ mutations are expected to weaken binding of eIF2(α P) as the means of overcoming inhibition of eIF2B, certain *gcn3^c* mutations in nearby residues likely inhibit eIF2B by strengthening an inhibitory mode of binding by unphosphorylated eIF2 to the eIF2B regulatory subcomplex.

Discussion

Archaeal aIF2B is a structural homolog of eIF2B regulatory subunits

Archaeal genomes encode many proteins annotated as orthologs of eIF2B regulatory subunits. However, this designation overlooks the fact that the eIF2B regulatory subunits are also related to the metabolic enzymes MTNA and RBPI. Indeed, we predict that ~50% of the 90 eIF2B-related archaeal proteins examined are MTNAs, based on (i) the presence of all 8 conserved sequence motifs present in *bone fide* MTNAs, including 6 presumptive active site residues 23²⁴, and (ii) a phylogenetic analysis (Fig. 2) that assigns these proteins exclusively to one monophyletic clade. Most archaeal genomes encode one such predicted MTNA. A second clade of eIF2B-related proteins, detected in ~50% of the species, includes the one documented RBPI from *T. kodakaraensis*. These last sequences contain all of the predicted active site residues of MTNAs but lack the N-terminal β -sheet and an 8-residue segment of motif IV. Finally, ~50% of the genomes encode a protein belonging to a third clade, which lack most of the conserved signature sequences of MTNAs and cluster on the phylogenetic dendrogram with eukaryotic eIF2B regulatory subunits (α , β , and δ), leading to our prediction that these proteins are archaeal orthologs of eIF2B subunits. Fortunately, this group includes the aIF2B of *P. horikoshii* PH0440, whose crystal structure was reported as a structural model for eIF2B α ²⁵, although our phylogenetic analyses indicates no closer relationship of PH0440 to eIF2B α than to eIF2B β or eIF2B δ (Fig. 2).

Consistent with our prediction, we found that members of the third clade (designated aIF2B) from *P. horikoshii*, *P. furiosus* and *T. acidophilum* all bound specifically to their cognate aIF2 α proteins *in vitro* and that the *T. acidophilum* aIF2B also bound yeast eIF2 α . Binding of *T. acidophilum* aIF2B to eIF2 α *in vitro* required only the N-terminal S1 domain of eIF2 α , and N-terminal deletions of this domain reduced binding of GST-eIF2 α to both aIF2B and the eIF2B regulatory subcomplex from yeast. This is consistent with previous findings that Gcn⁻ mutations in the S1 domain of eIF2 α reduce binding of phosphorylated GST-eIF2 α to the eIF2B regulatory subcomplex *in vitro* and reduce inhibition of eIF2B by eIF2(α P) *in vivo*^{9; 10}. Thus, it seems that the S1 domain of eIF2 α is a key binding site for both the eIF2B regulatory subcomplex and aIF2B. However, whereas eIF2B tightly binds only to Ser51-phosphorylated GST-eIF2 α , strong binding of *T. acidophilum* aIF2B to GST-eIF2 α was independent of phosphorylation and unaffected by Gcn⁻ mutations that weaken binding of phosphorylated GST-eIF2 α to the yeast eIF2B regulatory subcomplex. Thus, there appear to be important differences in the S1 domain contacts of aIF2B versus the eIF2B regulatory subcomplex, which are relevant to the mechanism that renders the eIF2B interaction with eIF2 α dependent on Ser51 phosphorylation in eukaryotes.

Although GST-aIF2B from *T. acidophilum* bound eIF2 α *in vitro* we found that over-expressing GST-aIF2B or untagged aIF2B did not complement the Gcn⁻ phenotype conferred by eliminating the α /GCN3 subunit of eIF2B in a *gcn3 Δ* mutant³⁴, nor the Gcd⁻ or Slg⁻ phenotypes of various *GCD2* or *GCD7* mutants with these phenotypes³⁵ (data not shown). Thus, it appears that aIF2B cannot functionally replace any of the three regulatory subunits of eIF2B *in vivo*. Interestingly, however, GST-aIF2B does form a stable complex with α /GCN3 in yeast cells and GST-aIF2B over-expression decreased the co-immunoprecipitation from WCEs of eIF2B regulatory subunits and eIF2 with the catalytic subunit (ϵ /GCD6) of eIF2B, consistent with destabilization of the eIF2B-eIF2 complex. As GST-aIF2B over-expression did not confer

dominant Slg^- , Gcn^- or Gcd^- phenotypes (data not shown), GST-aIF2B binding apparently did not sequester significant amounts of $\alpha/\text{GCN3}$ from eIF2B in living cells. Hence, we propose that *in vivo* GST-aIF2B binds to $\alpha/\text{GCN3}$ within the eIF2B-eIF2 complex without disrupting $\alpha/\text{GCN3}$ interactions with other eIF2B subunits or eIF2 α . We hypothesize that a GST-aIF2B $\alpha/\text{GCN3}$ heterodimer dissociates during the stringent washes applied to immobilized eIF2B-eIF2 complexes isolated from WCEs and this, in turn, provokes dissociation of the other regulatory subunits and eIF2 from the $\epsilon/\text{GCD6}$ - $\gamma/\text{GCD1}$ catalytic subcomplex. This is supported by the fact that the elimination of GCN3 in *gcn3 Δ* cells evokes a similar destabilization of immunoprecipitated eIF2B-eIF2 complexes.

Additional support for a physiologically significant aIF2B interaction with aIF2 α /eIF2 α was provided by the finding that aIF2 α co-purifies directly with His₁₀-HA-tagged aIF2B from *T. kodakaraensis* cells. Although aIF2 β and aIF2 γ also bound non-specifically to the Ni^{+2} resin used to purify His₁₀-tagged aIF2B, they also appeared to be present at higher abundance in the fractions containing His₁₀-tagged versus untagged aIF2B. Several 50S ribosomal subunit proteins also co-purified with His₁₀-aIF2B, findings arguing strongly that aIF2B functions in *T. kodakaraensis* cells in a complex involved in translation initiation. This is an interesting possibility, since eIF2B has been proposed to replace GTP when eIF2-GDP is bound to 60S ribosomes³⁶.

It is intriguing that a sugar-phosphate nucleotidyltransferase (TK0955) also copurified specifically with His₁₀-tagged aIF2B from *T. kodakaraensis* cells, as $\epsilon/\text{GCD6}$ and $\gamma/\text{GCD1}$ eIF2B subunits have nucleotide-binding (NUCT) and isoleucine-repeat (I-patch) domains in common with these enzymes³². The enzymes lack a critical domain found at the C-terminus of $\epsilon/\text{GCD6}$ that is needed for GEF activity. Moreover, the β -subunit of aIF2 lacks the N-terminal region that in eIF2 β binds eIF2B ϵ to promote nucleotide exchange²¹. Hence, it is improbable that TK0955 functions as the catalytic subunit of an archaeal GEF for aIF2. The roles of the NUCT and I-patch domains in the eIF2B catalytic subunits have not been determined, but it was suggested that the NUCT domain binds sugar or nucleotide allosteric regulators of eIF2B activity, and that the I-patch domain mediates heterodimerization between $\epsilon/\text{GCD6}$ and $\gamma/\text{GCD1}$ in the catalytic subcomplex³⁷. Our finding that aIF2B binds to TK0955 further implies that the NUCT or I-patch domains in the eIF2B catalytic subunits participate in protein-protein interactions with one or more of the regulatory subunits in eIF2B.

What is the function of aIF2B in Archaea? The interactions with aIF2 and 50S ribosomal proteins suggest that it regulates some aspect of aIF2 activity in translation initiation. Interestingly, the C-terminal domain (CTD) of eIF2 α is structurally similar to the CTD of eEF1B α , the GEF for translation elongation factor eEF1A, which functions analogously to eIF2 in the elongation phase of protein synthesis. It has been suggested that the CTD of aIF2 α might serve as an intrinsic GEF domain, and that the aIF2 α N-terminal domain (NTD) could regulate this function^{37, 38}. If so, then binding of aIF2B to the NTD of aIF2 α might stimulate (or inhibit) the intrinsic GEF activity of aIF2 α . This is an attractive possibility because aIF2B and its eukaryotic counterparts in eIF2B would then have analogous roles in modulating the respective GEFs for aIF2 and eIF2. We have deleted the gene (TK1047) that encodes aIF2B from the *T. kodakaraensis* genome and the deletion mutant grows normally under laboratory conditions (see supplementary material), demonstrating that aIF2B is not essential. This is not surprising, however, given that many archaeal genomes do not encode aIF2B orthologs (Table 1). Hence, if aIF2B regulates an intrinsic GEF activity of aIF2 α , this would have to be a function required only under certain physiological conditions, perhaps during stress or starvation.

Proposed structural model for the eIF2 α -P binding surface of the eIF2B regulatory subcomplex

Our findings that aIF2B can interact with aIF2 α /eIF2 α in vitro, with aIF2 in *T. kodakaraensis* cells, with eIF2B α /GCN3 in yeast cells, and with a *T. kodakaraensis* protein (TK0955) with NUCT and I-Patch domains found in eIF2B ϵ /GCD6, all suggest that the 3-D structure and surface properties of aIF2B are highly related to those of eIF2B regulatory subunits. Accordingly, we used the crystal structure of the *P. horikoshii* aIF2B homodimer to predict the locations of evolutionarily conserved residues and regulatory mutations in 3-D models of the eIF2B regulatory subunits. Our analysis predicts that each of the three subunits contains the majority of its evolutionarily conserved, solvent-exposed residues on the same face of the molecule, which also contains the majority of residues altered by Gcn⁻ mutations that render eIF2B insensitive to inhibition by eIF2(α P) and (at least for some) reduce binding of the eIF2B regulatory subcomplex to eIF2 α -P⁹. While spread over a large portion of the linear sequences of these proteins, the Gcn⁻ mutations demarcate limited regions of the conserved faces of the predicted C-terminal α/β domains, which are similar but non-identical in the three regulatory subunits. We propose that these regions represent the contributions of the regulatory subunits to a composite binding surface for phosphorylated eIF2 α .

How might eIF2B α , eIF2B β and eIF2B δ interact with one another in a heterotrimeric structure to arrange their conserved faces in one continuous surface? The crystal structure of the PH0440 homodimer depicted in Fig. 7A does not offer an obvious answer to this question. However, when viewed along the crystallographic 3-fold axis, it was noted previously that a trimer of PH0440 homodimers could interact to form a homohexamer (Fig. 10A), and it was proposed that this quaternary structure might provide a model for the heterotrimeric eIF2B regulatory subcomplex²⁵. The bulk of the available evidence indicates that the eIF2B-eIF2 complex contains one molecule of each of the eight different subunits^{39; 40; 41; 42}, which would preclude the occurrence of homodimers of the eIF2B regulatory subunits. However, it seems possible that a heterotrimer of eIF2B α , β , and δ monomers could assemble using the same interfaces employed in the predicted homohexameric structure, which would juxtapose the conserved faces of these three subunits to produce a continuous multivalent binding surface harboring the regulatory sites identified by Gcn⁻ mutations (Fig. 10B). We envision that this putative heterotrimer would be stabilized by interactions of its β and δ subunits with the ϵ and γ subunits of the catalytic subcomplex of eIF2B (Fig. 10B). Moreover, as virtually all of the eIF2B in yeast occurs in the form of eIF2B-eIF2⁴², interactions of eIF2 α with the regulatory subcomplex, and of eIF2 β and eIF2 γ with the catalytic subcomplex of eIF2B would further stabilize the whole assembly. We envision that α /GCN3's conserved surface interacts directly with the portion of the S1 domain of eIF2 α containing phosphorylated Ser51 (Fig. 10C). Interactions of the conserved surfaces in β /GCD7 and δ /GCD2 with other portions of the S1 domain, or even other regions of eIF2 α , would additionally be required for the tight binding of eIF2 α -P to the regulatory subcomplex that blocks productive interaction of the eIF2B ϵ catalytic domain with the GDP-binding pocket in eIF2 γ .

This model has several attractive features. First, it can accommodate the fact that eIF2B α /GCN3 is a nonessential eIF2B subunit in yeast, as its removal might weaken, but should not disrupt the remaining seven-subunit eIF2B-eIF2 co-complex. By envisioning direct interactions between the aIF2B-related domains in the β and δ regulatory subunits with the ϵ and γ catalytic subunits of eIF2B, the model incorporates our finding of interaction between aIF2B and the sugar-phosphate nucleotidyltransferase TK0955 in Archaea. The model also provides for a surface on α /GCN3 that would be available for interaction with aIF2B when the latter is overexpressed in yeast, as aIF2B could form a heterodimer with α /GCN3 in a manner mimicking aIF2B homodimerization (Fig. 10C). This interaction would not disrupt α /GCN3's interactions with other eIF2B subunits or eIF2 α in vivo, explaining the lack of Gcn⁻, Gcd⁻, or

Slg⁻ phenotypes associated with aIF2B overexpression in yeast. However, the α /GCN3-GST-aIF2B heterodimer could dissociate from the rest of the complex during the stringent washes applied to immobilized eIF2B-eIF2 complexes, as proposed above. To evaluate these and other predictions of our model, it will be necessary to determine the 3-D structure of a complex between the eIF2B regulatory subcomplex and eIF2 α -P. The results presented here indicate that analyzing the binary complex between aIF2B and aIF2 α could be a more tractable intermediate undertaking that could provide valuable insights into this intriguing structural problem.

Materials and Methods

Plasmids and yeast strains

The names of all plasmids used in this study, with brief descriptions and sources, are listed in Supplementary Table S1. Their constructions employed standard molecular biology techniques and were verified by restriction digests and sequencing, as described in the Supplementary Methods. The sequences of all primers employed are given in Table S2, except for primers 6009 and 6010 that were described previously³¹. The genes encoding aIF2 α and aIF2B were PCR amplified from samples of *Pyrococcus horikoshii*, *Pyrococcus furiosus*, and *Thermoplasma acidophilum* genomic DNA purchased from the ATCC (<http://www.atcc.org>).

Yeast strains H4 (*MAT α leu2-3 leu2-112 ura3-52*)³⁰ and H1331 (*MAT α leu2-3 leu2-112 ura3-52 gcn3::LEU2*)³⁴ were described previously, and transformed with plasmid DNA by standard methods⁴³.

GST pull-down assays with recombinant proteins expressed in *Escherichia coli*

Synthesis of GST-aIF2 α and His₆-aIF2B in *E. coli* BL21(DE3) (Novagen) harboring pPH0440-1, pPF0475-1, pTa0910-1, pPH096-1, pPF1140-1-1 or pTa1203-2 (Table S1) was induced by addition of 1 mM IPTG to 25 ml cultures growing in LB medium containing appropriate antibiotics at an OD₆₀₀ of 0.6-0.8. Incubation was continued at 37°C for 4 to 5 h to allow accumulation of His₆-aIF2B or GST-aIF2 α . Cells were harvested, resuspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM KCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT) containing protease inhibitors (Roche) and lysed by sonication (eight times for 30 s with 1 min intervals on ice). Whole cell-extracts (WCEs) were clarified by centrifugation at 13,000g for 20 min at 4°C.

For pull-down assays, glutathione-Sepharose beads (GE-Healthcare) were prewashed with binding buffer (BB; lysis buffer containing 0.1% Triton X-100; 5 mM NaF) and 30 μ l of a 50% slurry were mixed and incubated with a WCE containing a GST fusion protein for 2 h at 4°C on a rotator. The beads were washed three times with 500 μ l of ice-cold BB, and then incubated with a bacterial WCE containing a His₆-aIF2B protein for 4 h at 4°C in BB. To investigate salt dependency, NaCl was included in the binding buffer as indicated. After washing three times with 500 μ l of BB, the beads were resuspended in 20 μ l of 2 $\times$ Laemmli sample buffer. The polypeptides present were separated by electrophoresis through 4 to 20% SDS-polyacrylamide gradient gels (Biorad) and then transferred to a nitrocellulose membrane (Biorad) by electroblotting at 25 V for 2 h. The membrane was incubated with the appropriate antiserum, and the immune complexes formed were then visualized by incubation with a horseradish peroxidase-conjugated anti-rabbit secondary antibody and an enhanced chemiluminescence system (GE-Healthcare). For assays using phosphorylated GST-eIF2 α , the fusion proteins bound to glutathione-Sepharose beads were incubated with or without recombinant human PKR in kinase buffer, and the pull-down assays were performed as described previously¹⁰.

GST pull-down and co-immunoprecipitation assays using yeast WCEs

The yeast WCEs used for GST pull-down assays were prepared as described previously⁶ except that the cells were broken by vortexing with acid-washed glass beads five times for 1 min each, with 1 min intervals on ice, in 75 mM Tris-HCl (pH 7.5), 1 mM EDTA, with complete protease inhibitor tablets added to the buffer. Co-immunoprecipitation with anti-GCD6 antibodies was conducted as described previously⁴⁴; 7, except that 500 µg of WCE were used instead of the ribosomal salt wash fraction.

Epitope-tagging and deletion of TK1047 in *T. kodakaraensis*

A 2079 bp DNA fragment from the *T. kodakaraensis* chromosome containing the 3' terminus of TK1046 was PCR-amplified using primers B and C (Table S2). This DNA was digested with EcoRI and BamHI, and ligated with EcoRI- and BamHI-digested plasmid pUMT2 downstream of *trpE* to produce plasmid pH-1. A 2034 bp DNA fragment containing TK1047, TK1048 and the 5' terminus of TK1049 was amplified with primers D and E (Table S2), digested with PstI and HindIII, and ligated into PstI- and HindIII-digested pH-1 upstream of *trpE* to produce pH-2. QuikChange XL mutagenesis (Stratagene) was used to generate plasmids pH-3 and pH-4 from pH-2. Primers ΔTK1047-F and ΔTK1047-R were used to delete TK1047 from pH-2 to generate pH-3, and primers tagTK1047-F and tagTK1047-R were used to add HA-epitope and His₁₀ tag encoding sequences in-frame to the 3' terminus of TK1047 to generate pH-4. Aliquots of pH-2, pH-3 or pH-4 DNA were used to transform *T. kodakaraensis* strain KW128 (*ΔpyrF*; *ΔtrpE::pyrF*) as previously described⁴⁵. Transformants were selected by growth on plates lacking tryptophan, and were then grown in liquid MA-YT-S° medium overnight. Genomic DNA was isolated, and PCR amplification using primer pairs A and 6010, and F and 6009, were used to confirm that the desired integration had occurred into the *T. kodakaraensis* genome³¹. All constructs and derivatives were further verified by DNA sequencing. Representative *T. kodakaraensis* strains were designated H2, H3 and H4, respectively, that contained *trpE* inserted between TK1046 and TK1047, *trpE* inserted and TK1047 deleted, and *trpE* inserted plus TK1047-HA-his₁₀.

Computational methods

Sequence alignments (Fig. 1) were generated using Clustal W at <http://www.uniprot.org>, and multiple sequence alignments (Fig. 2) were generated as described⁴⁶. Phylogenetic analyses were carried out using the neighbor-joining method with nodal support assessed via bootstrapping (1000 replicates) as implemented in PAUP⁴⁷. The alignments in supplementary Figs. S1-S3 were obtained using Clustal W X version 2.0⁴⁸. ConSurf⁴⁹ and PyMOL⁵⁰ were used to obtain the sequence conservation scores and generate the surface representations of sequence conservation, respectively, shown in Figs. 7, 8 and 9.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIG. 1. Alignment of archaeal sequences related to eIF2B regulatory subunits demonstrating distinct protein families

A multiple sequence alignment was constructed using ClustalW for 24 archaeal sequences retrieved in a BLAST search using PH0440 as the query sequence. The sequences are identified on the far left by the abbreviations of their species of origin (listed below) followed by the UniProtKB/Swiss-Prot accession numbers. As explained in the text, the sequences have been grouped according to their classification as RBPI-like (top 8), MTNA-like (middle 9), or aIF2B-like (bottom 7) sequences, based on the presence (or absence) of the following sequence motifs (*mI* to *mVIII*) which are highly conserved in eukaryotic and bacterial MTNAs: [QxxLP]_I, [VRGAPx]_{II}, [LxxRPTA]_{III}, [TxCNxGxxATxxxGTA]_{IV}, [ETRPxxQGxxLTxxE]

(x)₁₂D]_V; [GAD(x)₅GDxANKxGTxxLA(x)₉F]_{VI}; [GxxIxxExRxxxEx(x)₅G]_{VII}; and [FDxTPxxLI(x)₇G]_{VIII}^{23; 24} The locations of *mI* to *mVIII* are indicated on the line immediately below the MTNA-like sequences. The underlined residues in the motifs are proposed to make contacts with the bound phosphate in yeast MTNA²³. Residues in the archaeal sequences matching the motifs are shown in red. Positions within these motifs that are identical, highly conserved or moderately conserved in all 24 archaeal sequences are indicated below the aIF2B-like sequences with asterisks, colons or periods, respectively. The species abbreviations are: ARCFU, *Archaeoglobus fulgidus*; HALMA, *Haloarcula marismortui*; METJA, *Methanococcus jannaschii*; META3, *Methanococcus aeolicus*; METHJ, *Methanospirillum hungatei*; PYRFU, *Pyrococcus furiosus*; PYRHO, *Pyrococcus horikoshii*; TKO, *Thermococcus kodakaraensis*; PYRAB, *Pyrococcus abyssi*; THEAC, *Thermoplasma acidophilum*.

FIG. 2. Phylogenetic analysis of proteins related to eIF2B regulatory subunits identifies putative aIF2B orthologs

The phylogenetic dendrogram was constructed using the neighbor-joining method as implemented by PAUP from an alignment of the archaeal sequences listed in Table 1 as MTNA-like (aMTNA), RBPI-like (aRBPI), or aIF2B-like (aIF2B), together with sequences of eukaryotic MTNAs (eMTNA) and eIF2B regulatory subunits (eIF2B α , eIF2B β , or eIF2B δ) identified in Supplementary Table S3. Significant bootstrap values (>70) are shown above the branches. The abbreviations used to identify archaeal sequence origins are defined in Table 1. The eukaryotic sequences are identified as follows: At: *Arabidopsis thaliana*; Os: *Oryza*

sativa; Sc: *Saccharomyces cerevisiae*; Sp: *Schizosaccharomyces pombe*; Af: *Aspergillus fumigatus*; Dm: *Drosophila melanogaster*; Hs: *Homo sapiens*.

FIG. 3. Archaeal aIF2α proteins bind their cognate aIF2Bs *in vitro*

(A) Aliquots of *E. coli* WCE containing GST-aIF2α from *T. acidophilum* (300 and 400 μg, lanes 2 and 3), *P. horikoshii* (200, 300 or 400 μg, lanes 8-10), or *P. furiosus* (200, 300, or 400 μg, lanes 16-18) or equal amounts of GST alone (lanes 5 and 6, 12-14, 20-22) were incubated with glutathione-Sepharose beads to immobilize the GST proteins. After washing, the beads were incubated with 400 μg bacterial WCE containing the His-aIF2B of the corresponding species. After extensive washing, the bound polypeptides (P, pellet) were separated by SDS-PAGE and analyzed by Western blotting using antibodies against GST or the hexa-histidine tag. Lanes 1, 4, 7, 11, 15, and 19 contain 5% of the total input (I) amounts of WCEs used for the reactions. (B) *T. acidophilum* GST-aIF2α or GST alone from 400 μg of bacterial WCE

were immobilized on glutathione-Sepharose beads and incubated with 400 μ g WCE from cells containing the vector plasmid (P, lane 4) or a plasmid expressing His₆-aIF2B (P, lane 5), as in (A). Bound proteins were resolved by SDS-PAGE and visualized by Coomassie Blue (CBB-R250) staining. Lanes 1-3 contain 10% of the input (I) fractions for GST alone, GST-aIF2 α , and His-aIF2B respectively. Stars indicate the respective full-length proteins, as confirmed by Western blotting. Molecular size markers (kDa) are indicated on the right. (C) GST-aIF2 α from *P. horikoshii* (lanes 4-11) or GST alone (lanes 12-17) from 300 or 400 μ g of *E. coli* WCEs were immobilized on glutathione-Sepharose beads and incubated with 400 μ g bacterial WCE containing *P. horikoshii* His-aIF2B in the presence of 100, 200 or 300 mM NaCl, as indicated. Bound proteins (Pellet) were analyzed as in (A). Lanes 1-3 contain 5% of the input (I) amounts of WCEs used for the reactions.

FIG. 4. N-terminal S1 domain of yeast eIF2α is involved in the interaction with *T. acidophilum* aIF2B and the yeast eIF2B regulatory subcomplex

(A) Ribbons depiction of the co-crystal structure of human PKR (orange) and the N-terminal amino acids 3-175 of region of yeast eIF2α (PDB ID 2a19). Strands β1-β5 of the S1 domain of eIF2α are colored blue, black, green, red, and yellow, respectively. The locations of Gcn⁻ mutants R88T and L84F, analyzed in (Fig. 5B) are indicated. (B) Full-length wild-type GST-eIF2α (WT 1-304 aa) and the indicated derivatives truncated at the C terminus (designated by the amino acids [aa] remaining) were immobilized on glutathione-Sepharose beads and assayed for binding *T. acidophilum* His-aIF2B as described for Fig. 3A, except that 100, 200, or 300 μg of WCE containing the GST fusions were employed. Lanes 1, 5, 9, 13, and 17 contain 5% of total input (for the highest amounts of WCE containing GST fusions) used in the assays (C) GST pull down assays conducted as in (B) except using 200 or 300 μg of WCE containing WT GST-eIF2α or the indicated derivatives truncated at the N terminus (designated by the amino acids remaining). The star indicates GST produced by partial degradation of the GST-eIF2α fusions visible in lanes 1-6. (D) WT GST-eIF2α (lanes 6-9), the indicated truncations described in (B or C) (lanes 10-17), or GST alone (lanes 18 and 19) in two different amounts (200 and 400 μg) of bacterial extract were immobilized on glutathione-Sepharose beads and incubated

with (+) or without (-) purified PKR in kinase assay buffer. After washing, immobilized proteins were incubated with 500 μ g of WCE from yeast strain H4 over-expressing all three eIF2B regulatory subunits from a high-copy plasmid (HC 2/7/3). After washing, the bound proteins (Pellet, lanes 6-19) were analyzed by Western blotting using antibodies against GCD2, GCD7, GCN3, GST, or Ser51-phosphorylated eIF2 α , as indicated. Lane 1 contains 10% of the input yeast WCE, whereas lanes 2-5 contain 5% of the total input (I) amounts of bacterial WCEs containing the GST proteins. Species in lanes 12-14 detected with GCD2 and GCD7 antibodies do not exhibit the same mobilities as GCD2 and GCD7 in lanes 6-7 and, hence, are regarded as non-specific, cross-reacting species.

A

B

FIG. 5. Neither phosphorylation of Ser-51 by PKR nor Gcn⁻ mutations affect interaction of eIF2α with *T. acidophilum* aIF2B

(A) Wild-type GST-eIF2α in three different amounts (200, 300 and 400 μg) of bacterial WCE were immobilized on glutathione-Sepharose beads and phosphorylated by PKR (+), or left unphosphorylated (-), before incubating with either 500 μg of yeast WCE containing the over-expressed eIF2B regulatory subunits or 400 μg bacterial WCE containing *T. acidophilum* His-aIF2B, and the bound proteins were detected by Western blot analysis, all as described in Figs. 4B and D. Lanes headed by P contain the bound fractions. Lanes 1 and 5 contain 10%, whereas lanes 9 and 13 contain 5%, of the input (I) WCEs. (B) Pull-down assays were done as described in Fig. 4B, except that 200 or 300 μg of WCEs containing either WT GST-eIF2α, GST-eIF2α X-R88T, GST-eIF2α-L84F, or GST alone and 400 μg of WCE containing *T. acidophilum* His-aIF2B were used. Lanes 1-5 contain 5% of the input (I) amounts of WCE.

FIG. 6. *T. acidophilum* aIF2B interacts with yeast eIF2B α /GCN3 in vivo and destabilizes the eIF2·eIF2B complex in vitro

(A) Transformants of yeast strain H4 harboring plasmids pGAL-Ta0910 or pEGKT encoding, respectively, *T. acidophilum* GST-eIF2B or GST alone under the *GAL* promoter, were grown at 30°C to A₆₀₀ of ~ 0.8 in SC medium containing 2% raffinose as carbon source and shifted to SC medium containing 2% raffinose and 2% galactose and an additional 4 h of incubation. The GST-eIF2B or GST proteins in three different amounts (200, 300 and 400 µg) of yeast WCE were immobilized on glutathione-Sepharose beads. After extensive washing, bound proteins (Pellet, lanes 2-4 and 6-8) were analyzed by Western blotting using antibodies against eIF2B subunits GCD6, GCD1, GCD2, GCD7, and GCN3, eIF2α, eIF2β, eIF2γ, and GST, as

indicated. Lane 1 and 5 contains 10% of the input (I) amounts of WCE. (B) Yeast WCE (500 μ g) from *gcn3* Δ strain H1331 grown in SC medium and the WCEs from (A) were immunoprecipitated using polyclonal antibodies against GCD6 and protein A Sepharose beads. After extensive washing, immunocomplexes (P, lanes 2, 4 and 6) were analyzed by Western blotting using antibodies against GCD6, GCD1, GCD2, GCD7, GCN3, and eIF2 α . Lanes 1, 3 and 5 represent 10% of the input (I) amounts. (C) Alignment of the NUCT domains in eIF2B ϵ /GCD6, TK0955, and the consensus NUCT domain sequence defined previously³² using the following symbols: (.) any residue allowed; U or u, highly or moderately conserved bulky hydrophobic residue, respectively; O or o, highly or moderately conserved small residue; J or j, highly or moderately conserved positively charged residue; \$, Ser or Thr. Identities (|) and conservative replacements (*) are indicated.

FIG. 7. Predicted surface conservation of eIF2B regulatory subunits

(A) Ribbons depiction of the crystal structure of the anti-parallel homodimer of *P. horikoshii* eIF2B, PH0440 (PDB ID 1vb5). The black line indicates the dimer interface between the two protomers. The N-terminal domain (NTD, residues 1-95) is connected to C-terminal domain (CTD, residues 96-276) by a long $\alpha 5$ -helix (residues 78-106). In (II), on the right-hand side, the dimer was rotated 180° about the vertical axis from the view in (I). (B). Model predicting the sequence conservation of surface-exposed residues among eIF2B β /GCD7 homologs. Highly conserved residues are colored in red shades, whereas highly variable residues are colored in green shades as indicated in the conservation scale at the very bottom. Multiple sequence alignment of eIF2B β /GCD7 homologs and PH0440 was performed (supplementary

Fig. S1) and the degree of sequence conservation at amino acid positions that can be aligned with the PH0440 sequence is projected on the surface of the PH0440 homodimer. Highly conserved residues (red) are clustered on one surface (I), whereas most of the variable residues (blue) are on the opposite face (II). (C) and (D). Models predicting the sequence conservation of surface-exposed residues among eIF2B δ /GCD2 (C) and eIF2B α /GCN3 homologs, constructed from the alignments shown in Figs. S2 and S3, respectively, just as in (B).

FIG. 8. Spontaneous Gcn^- mutations are enriched on the predicted conserved face of eIF2B β /GCD7 (A). The image on the left shows the predicted sequence conservation on the conserved face of the hypothetical β /GCD7 homodimer, exactly as depicted in Fig. 7B, with the addition of stars indicating the positions of single amino acid substitutions conferring a Gcn^- phenotype in yeast ⁷. The locations of these Gcn^- mutations are shown in shades of blue on the model on the right, which depicts the same view of the homodimer but without sequence conservation coloration. The amino acid substitutions in β /GCD7 are given in blue type in parentheses under the corresponding PH0440 residues in black type. Pairs of residues colored black on the model are at the borders of inserts found in eIF2B β /GCD7 (*i-1* to *i-4*) with no corresponding sequences in PH0440 (see alignments in Fig. S1). These inserts contain the Gcn^- mutations listed in parentheses in blue type. Note that amino acid residues have been colored blue or black in both protomers, but labeled in only one of the two protomers. (B-C). The β /GCD7 homodimer model in (A) has been rotated successively 90° about the vertical axis to show the locations of the two Gcn^- mutations that do not map to the highly conserved face visible in (A). The view in (C), designated II, is identical to view II in the right-hand panels of Fig. 7.

FIG. 9. Spontaneous Gcn^- mutations are enriched on the predicted conserved faces of $eIF2B\delta$ /GCD2 and $eIF2B\alpha$ /GCN3, as are Gcd^- mutations in $eIF2B\alpha$ /GCN3
 (A-B) The locations of spontaneous Gcn^- mutations in yeast δ /GCD2⁷ are depicted in views I and II, identical to those shown in Fig. 7C, following the same conventions described in Fig. 8 for β /GCD7 mutations. (C-D) The locations of Gcn^- (blue) and Gcd^- (orange) mutations in yeast α /GCN3⁷; 34 are depicted in views I and II, identical to those shown in Fig. 7D, following the same conventions described in Fig. 8 for β /GCD7 mutations. Surface conservation on the left panel is as shown in Fig. 7D. The amino acid residues of GCN3 which produce Gcd^- phenotype are colored in orange shades, whereas the one that correspond to Gcn^- phenotype are colored in blue (right panel). The $eIF2B$ amino acid residues are numbered in black,

followed by yeast mutations in parenthesis. (D) The dimer has been rotated 180° about the vertical axis in opposite direction showing the least conserved face.

FIG. 10. Hypothetical structural model for eIF2B and its interactions with eIF2 α -P and aIF2B
 (A) Homohexameric structure of PH0440 viewed from below along the crystallographic 3-fold axis (reproduced from 25). The three homodimer interfaces labeled "1" are identical to those indicated in Fig. 7; those labeled "2" represent interactions among the three homodimers, involving the long helix $\alpha 5$ in each protomer, that would stabilize the proposed homohexamer.
 (B) Hypothetical model for eIF2B. The aIF2B-related domains of δ /GCD2, β /GCD7, and α /GCN3 are depicted in surface representations with sites of Gcn⁻ mutations colored blue and Gcd⁻ mutations colored orange, as in Figs. 8A, 9A and 9C, and arranged in the manner of the three aIF2B protomers delineated in panel A by dotted outlining, to produce a hypothetical regulatory subcomplex. The subunits γ /GCD1 and ϵ /GCD6 of the catalytic subcomplex interact

with the δ /GCD2 and β /GCD7 subunits of the regulatory subcomplex. (C) The same model in panel B but showing the predicted binding surface for eIF2 α on the conserved face of the eIF2B regulatory subcomplex and the proposed heterodimer formation between aIF2B and α /GCN3 using the dimer interface “1” defined in (A). (see text for further details.)

Table 1
Predicted protein sequences similar to eIF2B regulatory subunits in various archaeal species¹

Species	Archaeal lineage ²	MTNA-like	RBPI-like	aIF2B-like
<i>Thermofilum pendens</i> (THEPD)	C	A1RWS6 (Tpen_0246)	A1RX63(Tpen_0384)	A1RX64 (Tpen_0385)
<i>Hyperthermus butylicus</i> (HYPBU)	C	A2BLE3 (Hbut_0956)	A2BK49 (Hbut_0498)	A2BK50 (Hbut_0499)
<i>Staphylothermus marinus</i> (STAMF)	C	A3DN77 (Smar_0989)	A3DP88 (Smar_1357)	A3DP89 (Smar_1358)
<i>Aeropyrum pernix</i> (AERPE)	C	Q9YE84 (APE_0686)	—	Q9YD65 (APE1047)
<i>Caldivirga maquilingensis</i> (CALMQ)	C	A8MBP9 (Cmaq_0396)	—	A8MBN4 (Cmaq_1951)
<i>Pyrobaculum arsenaticum</i> (PYRAR)	C	A4WGV8 (Pars_0008)	—	A4WHN4 (Pars_0288)
<i>Pyrobaculum aerophilum</i> (PYRAE)	C	Q8ZYJ8 (PAE0741)	—	Q8ZXJ8 (PAE1244)
<i>Pyrobaculum islandicum</i> (PYRIL)	C	A1RSA9 (Pisl_0663)	—	A1RU20 (Pisl_1289)
<i>Pyrobaculum caldifontis</i> (PYRCI)	C	A3MY71 (Pcal_2173)	—	A3MYY1 (Pcal_1377)
<i>Thermoproteus neutrophilus</i> (THENV)	C	BIY9P1 (Tneu_0005)	—	BIYB83 (Tneu_0261)
<i>Cenarchaeum symbiosum</i> (CENSY)	C	A0RV57 (CENSYa_0591)	—	—
<i>Sulfolobus solfataricus</i> (SULSO)	C	Q9TUM6 (SSO2978)	—	—
<i>Sulfolobus tokodaii</i> (SULTO)	C	Q973H9 (ST0922)	—	—
<i>Sulfolobus acidocaldarius</i> (SULAC)	C	Q4JB92 (Saci_0539)	—	—
<i>Pyrococcus furiosus</i> (PYRFU)	E	Q8U178 (PF1349)	Q8U4G6 (PF0122)	Q8U3J1 (PF0475)
<i>Pyrococcus horikoshii</i> (PYRHO)	E	O58433 (PH0702)	O57947 (PH0208)	O58185 (PH0440)
<i>Thermococcus kodakaraensis</i> (TKO)	E	Q5JF43 (TK0556)	Q5JFM9 (TK0185)	Q5JDZ9 (TK1047)
<i>Pyrococcus abyssi</i> (PYRAB)	E	Q9UZ16 (PYRAB13410)	Q9V281 (PYRAB01930)	Q9UYB6(PYRAB15920)
<i>Methanococcus jannaschii</i> (METJA)	E	Q57896 (MJ0454)	Q57586 (MJ0122)	—
<i>Methanococcus aeolicus</i> (META3)	E	A6USZ8 (Maeo_0027)	A6UUT5 (Maeo_0673)	—
<i>Methanoculleus marisnigri</i> (METMI)	E	A3CW71 (Memar_1694)	A3CX93 (Memar_2067)	—
<i>Methanoregula boonei</i> (METB6)	E	A7I9E4 (Mboo_1840)	A7IB35 (Mboo_2432)	—
<i>Methanoseta thermophila</i> (METTP)	E	A0B634 (Mthe_0365)	A0B8L7 (Mthe_1262)	—
<i>Methanosarcina acetivorans</i> (METAC)	E	Q8TUJ1 (MA_0076)	Q8TTP7(MA_0379)	—
<i>Methanocorpusculum labreanum</i> (METLZ)	E	A2SS42 (Mlab_0978)	A2SQX5 (Mlab_0557)	—
<i>Methanosarcina barkeri</i> (METBF)	E	Q46DN3 (Mbar_A1041)	Q46EC2 (Mbar_A0793)	—
<i>Methanosarcina mazei</i> (METMA)	E	Q8PX51 (MM_1371)	Q8PW10 (MM_1606)	—
<i>Methanococcoides burtonii</i> (METBU)	E	Q12U85 (Mbur_2118)	Q12UQ7 (Mbur_1938)	—
<i>Archaeoglobus fulgidus</i> (ARCFU)	E	O29877 (AF_0370)	O28242 (AF_2037)	—

Species	Archaeal lineage ²	MTNA-like	RBPI-like	aIF2B-like
<i>Methanospirillum hungatei</i> (METHJ)	E	Q2FN46 (Mhun_0623)	Q2FSY5 (Mhun_2314)	—
<i>Methanobacterium thermoautotrophicum</i> (METTH)	E	O27900 (MTH_1872)	—	—
<i>Methanobrevibacter smithii</i> (METS3)	E	A5ULDI(Msm_0804)	—	—
<i>Methanococcus maripaludis</i> (METMP)	E	Q6L-WT8 (MMP1618)	—	—
<i>Methanosphaera stadtmanae</i> (METST)	E	Q2NFI6 (Msp_1023)	—	—
<i>Methanococcus maripaludis</i> (METM7)	E	A6VHK6 (MmarC7_0865)	—	—
<i>Methanopyrus kandleri</i> (METKA)	E	Q8TYG4 (MK0335)	—	—
<i>Methanococcus vannielii</i> (METVS)	E	A6UQQ9 (Mevan_0927)	—	—
<i>Thermoplasma acidophilum</i> (THEAC)	E	Q9HIW4 (Ta1212)	—	Q9HIQ7 (Ta0910)
<i>Thermoplasma volcanium</i> (THEVO)	E	Q978Y9 (TV1275)	—	Q97A32 (TV0978)
<i>Picrophilus torridus</i> (PICTO)	E	Q6L2F6 (PTO0261)	—	Q6L1P0(PTO0527)
<i>Halobacterium salinarum</i> (HALSA)	E	—	Q9HP11 (VNG1853G)	Q9HPV9 (VNG1452G)
<i>Halorubrum lacusprofundi</i> (HALLT)	E	—	B9LRX1 (Hlac_2269)	B9LR Y1 (Hlac_2279)
<i>Natrialba magadii</i> (NATMA)	E	—	B9ZFW1 (NmagDRAFT_1314)	B9ZEY5 (NmagDRAFT_1705)
<i>Natronomonas pharaonis</i> (NATPD)	E	—	Q3IQ44 (NP2020A)	Q3ITB8(NP1050A)
<i>Halobacula marismortui</i> (HALMA)	E	—	—	Q5V0X9 (rmAC1956) Q5V557 (rmAC0289) Q5V075 (rmAC2247)
<i>Halobacterium walsbyi</i> (HALWD)	E	—	—	Q18GS5 (HQ2713A)
Uncultured methanogenic archaeon (UNCMA)	E	Q0W2L6 (UNCMA_08750)	Q0W0H9 (UNCMA_01520)	—

¹ A BLAST search of the archaeal genome database was conducted using *P. horikoshii* aIF2B (PH0440; Swiss-Prot O58185) and the 89 sequences with the highest BLAST scores were subjected to multiple sequence alignments using ClustalW and classified according to the presence or absence of MTNA signature motifs ³⁶, labeled *mI-mVIII*, as illustrated for a subset of the sequences in Fig. 1. MTNA-like sequences contain perfect or nearly perfect matches to all eight motifs. RBPI-like sequences also contain perfect matches to the MTNA motifs except for an N-terminal truncation that removes *mI* and an 8- residue deletion in *mIV*. The aIF2B-like proteins also exhibit these two differences from MTNA-like sequences and also contain degenerate versions of the other motifs. (See Fig. 1 and text for more details.) Sequences are designated by their Swiss-Prot accession numbers and ordered locus numbers (shown in parenthesis).

² E = *Euryarchaea*; C = *Crenarchaea*

Table 2
MudPit analysis of proteins co-purifying with *T. kodakaraensis* His₁₀-HA-tagged aIF2B¹

Orderd locus no.	Interacting protein	MASCOT score	no. of peptides matched (% sequence coverage)
TK1047	Translation initiation factor IF-2B alpha	1209	28 (60)
TK1557	Dehydrogenase	444	12 (13)
TK1502	50S ribosomal protein L18e	404	10 (33)
TK0137	Nucleic acid binding protein	404	12 (47)
TK0955	Sugar-phosphate nucleotidyltransferase	283	8 (11)
TK1417	50S ribosomal protein L1P	254	5 (30)
TK1100	Translation initiation factor IF-2 alpha	236	9 (8)
TK1522	50S ribosomal protein L18P	224	6 (21)
TK1520	50S ribosomal protein L30P	200	5 (16)
TK1410	DNA primase	192	6 (9)
TK1541	50S ribosomal protein L4P	170	4 (6)
TK2200	Hypothetical protein	167	6 (10)
TK1528	50S ribosomal protein L5P	163	7 (20)
TK2304	50S ribosomal protein L2P	162	4 (7)
TK1542	50S ribosomal protein L3P	151	4 (12)
TK0477	HAD superfamily hydrolase	148	4 (10)
TK0019	ABC transporter ATPase	136	2 (3)
TK1636	Putative RNA-associated protein	115	2 (4)
TK1418	50S ribosomal protein L11P	111	2 (7)

¹Proteins with MASCOT scores of greater than 100 identified in the eluate containing His₁₀-HA-tagged aIF2B from the H4 (tagged) strain of *T. kodakaraensis* are listed in descending order, along with the number of peptides matched to the relevant protein and the percentage of its amino acid sequence covered by the matching peptides. None of the listed proteins were detected in equivalent column fractions prepared from the untagged H2 strain (See text for further details).