Homocysteine accumulation causes a defect in purine biosynthesis: further characterization of *Schizosaccharomyces pombe* methionine auxotrophs

Yasuko Fujita,^{1,2} Eiko Ukena,³ Haruyuki Iefuji,³ Yuko Giga-Hama² and Kaoru Takegawa¹

Correspondence
Kaoru Takegawa
takegawa@ag.kagawa-u.ac.jp

¹Department of Life Sciences, Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa 761-0795, Japan

²Research Center, Asahi Glass Co. Ltd, Yokohama, Kanagawa 221-8755, Japan

³National Research Institute of Brewing, Higashi-Hiroshima, Hiroshima 739-0046, Japan

Methionine synthase (EC2.1.1.14) catalyses the final step in methionine synthesis, i.e. methylation of homocysteine. A search of the *Schizosaccharomyces pombe* genomic database revealed a gene designated SPAC9.09, encoding a protein with significant homology to methionine synthase. Disruption of SPAC9.09 caused methionine auxotrophy, and thus the gene was identified as a methionine synthase and designated *met26*. The *met26* mutant was found to exhibit a remarkable growth defect in the absence of adenine even in medium supplemented with methionine. This phenotype was not observed in other methionine auxotrophs. In the budding yeast *Saccharomyces cerevisiae*, which has been reported to utilize homocysteine in cysteine synthesis, lack of a functional methionine synthase did not cause a requirement for adenine. The introduction of genes from *Sac. cerevisiae* constituting the cystathionine pathway (*CYS4* and *CYS3*) into *Sch. pombe* $\Delta met26$ cells restored growth in the absence of adenine. HPLC analysis showed that total homocysteine content in $\Delta met26$ cells was higher than in other methionine auxotrophs and that introduction of the *Sac. cerevisiae* cystathionine pathway decreased total homocysteine levels. These data demonstrate that accumulation of homocysteine causes a defect in purine biosynthesis in the *met26* mutant.

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INTRODUCTION

Biosynthesis of sulfur-containing amino acids has been studied extensively at the genetic, enzymic and regulatory levels in various micro-organisms because of interest in gene regulation, the complexity of the pathway and the important roles sulfur amino acids play in metabolism (Paszewski, 1993; Marzluf, 1997; Thomas & Surdin-Kerjan, 1997).

In the fission yeast *Schizosaccharomyces pombe*, biosynthesis of cysteine and related metabolites has been examined extensively at the enzymic level. Brzywczy *et al.* (2002) reported that *Sch. pombe* possesses a specific variant pathway for *de novo* biosynthesis of cysteine. Whereas the budding yeast *Saccharomyces cerevisiae* synthesizes cysteine via the cystathionine pathway (homocysteine-cystathionine-cysteine; Fig. 1A, e and f), *Sch. pombe* synthesizes cysteine via the *O*-acetylserine pathway (serine-*O*-acetylserine-cysteine; Fig. 1B, g and h). We generated a cysteine auxotroph by blocking the *O*-acetylserine pathway as a means of investigating biosynthesis in *Sch. pombe* and have shown that *cys1a* encodes cysteine synthase, which catalyses

the last step in the *O*-acetylserine pathway (Fig. 1B, h). As expected, a *cys1a* mutant exhibited cysteine auxotrophy (Fujita & Takegawa, 2004). In contrast to the cysteine biosynthetic pathway, methionine biosynthesis in *Sch. pombe*, including the steps of sulfur assimilation, have not been studied in great detail at either the enzymic or genetic level. While *met6* has been identified as encoding homoserine *O*-acetyltransferase (Schweingruber *et al.*, 1998) (Fig. 1, c), other genes involved in the methionine biosynthetic pathway are still unidentified.

In this report, the *Sch. pombe* methionine synthase gene (designated *met26*), which encodes the enzyme catalysing the last step in methionine biosynthesis (Fig. 1, d), was characterized. A *met26* mutant exhibited not only methionine auxotrophy but also a growth requirement for adenine, i.e. a purine requirement. In contrast, other methionine auxotrophic mutants did not require adenine. Furthermore, a purine requirement was not observed in a methionine synthase mutant of *Sac. cerevisiae*. These data suggest that the purine requirement of the *Sch. pombe met26*

METHODS

generated as follows. ARC039 was converted into an arginine and histidine auxotroph by disruption of *arg12* and *his2*, respectively. Gene disruption was performed by repeated *ura4* insertion into a target gene and subsequent positive selection using 5'-fluoroorotic acid. *Δade7* was generated by disrupting *ade7* of ARC039 as described by Fujita *et al.* (2005). *Sac. cerevisiae* strains BY4742 and *met6* (Clone ID 16396) were obtained from Open Biosystems. Synthetic minimal medium (MM) for *Sch. pombe* cells was used as described by Alfa *et al.* (1993). Basal MM medium was supplemented with 240 μg leucine ml⁻¹, 75 μg ml⁻¹ each of uracil, arginine, lysine, histidine and glutamic acid. *Sac. cerevisiae* strains were grown in synthetic defined (SD) medium (Wickerham, 1946). Basal SD medium was supplemented with 100 μg ml⁻¹ each of leucine, uracil, lysine, histidine and tryptophan. A previously described electroporation method (Suga & Hatakeyama, 2001) was used to disrupt genes

Table 1. Strains used in this study

Strain	Genotype	Source
<i>Sch. pombe</i>		
ARC039	<i>h⁻ leu1-32 ura4-M190T</i>	Asahi Glass Co.
$\Delta met26$	<i>h⁻ leu1-32 ura4-M190T met26::ura4</i>	This study
$\Delta met14$	<i>h⁻ leu1-32 ura4-M190T met14::ura4</i>	This study
$\Delta met16$	<i>h⁻ leu1-32 ura4-M190T met16::ura4</i>	This study
$\Delta met6$	<i>h⁻ leu1-32 ura4-M190T met6::loxP</i>	This study
$\Delta ade7$	<i>h⁻ leu1-32 ura4-M190T ade7::ura4</i>	This study
YF031	<i>h⁻ leu1-32 ura4-M190T arg12::ura4 his2::ura4 met26::ura4</i>	This study
YF041	<i>h⁻ leu1-32 ura4-M190T arg12::ura4 his2::ura4 met26::ura4 ade7::ura4</i>	This study
<i>Sac. cerevisiae</i>		
BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	Open Biosystems
<i>met6</i>	BY4742, <i>met6::KAN</i>	Open Biosystems

Table 2. List of genes characterized in this study

<i>Sac. cerevisiae</i> gene name	Pathway in Fig. 1	<i>Sch. pombe</i> homologue	Gene product
<i>MET6</i>	d	SPAC9.09 (<i>met26</i>)	Methionine synthase
<i>MET14</i>	a	SPAC1782.11 (<i>met14</i>)	Adenylylsulfate kinase
<i>MET16</i>	b	SPAC13G7.06 (<i>met16</i>)	3'-Phosphoadenylylsulfate reductase
<i>MET2</i>	c	<i>met6</i>	Homoserine O-acetyltransferase

in *Sch. pombe* and a modified lithium acetate method (Morita & Takegawa, 2004) was used for plasmid transformation. *Escherichia coli* XL-1 Blue (Stratagene) was used for all cloning procedures.

Gene disruptions. A list of genes characterized in this study is given in Table 2. *Sch. pombe met26*, *met14* and *met16* were disrupted using *ura4* as a selective marker (Grimm *et al.*, 1988). The 2.9, 0.85 and 0.98 kb DNA fragments, including the whole or a part of the ORF of *met26*, *met14* and *met16*, respectively, were amplified from wild-type *Sch. pombe* genomic DNA and subcloned into pGEM-T Easy (Promega). *met26* was disrupted by elimination of a 1.16 kb *HindIII*–*HindIII* fragment from the ORF followed by insertion of a *ura4* cassette. *met14/met16* was disrupted by insertion of a *ura4* cassette into a *HindIII* site within a subcloned ORF fragment. Linearized DNA fragments carrying disrupted genes were used to transform parent strain ARC039 and stable *ura4* transformants were selected. Genomic disruption of target genes was confirmed by PCR. Primers used are listed in Table 3. A *met26* mutant, YF031, carrying arginine and histidine auxotrophies was also generated by disrupting *met26* in strain YF023. Furthermore, YF041 was generated by disrupting *ade7* in YF031.

A *loxP*-flanked *ura4* cassette was used to disrupt *Sch. pombe met6*. DNA fragments for gene disruptions were constructed using the *loxP* cassette vector pBS *loxP-ura4-loxP* (Iwaki & Takegawa, 2004) as follows. Fragments (0.6 or 0.5 kb) of the upstream or downstream region of the *met6* ORF were amplified from wild-type *Sch. pombe* genomic DNA. Upstream fragments were amplified using a sense primer carrying an *XbaI* site and an antisense primer carrying a *BamHI* site. Downstream fragments were amplified using a sense primer carrying a *HindIII* site and an antisense primer carrying an *XhoI* site (see Table 3). Amplified fragments were digested with the corresponding restriction enzymes and the *XbaI*–*BamHI* upstream fragment and *HindIII*–*XhoI* downstream fragment were inserted on either side of the *loxP-ura4-loxP* cassette. Linearized DNA fragments carrying the upstream and downstream region flanking the *loxP-ura4-loxP* cassette were used to transform parent strain ARC039. Disruption of the target gene was confirmed by PCR. Mutants were subsequently transformed with the Cre recombinase vector pRep41–Cre. Transformants were subcultured twice on YES plates and several clones that had lost *LEU2* activity were picked. Most had lost *ura4* activity through elimination of the marker due to *loxP* site-specific recombination.

Plasmid construction. pAL-*met26* was constructed by integrating full-length *met26* into the *NotI* site of the multi-cloning site (MCS) of pAL-SK (+). pAL was a gift from Dr C. Shimoda (Osaka City University, Japan). A *met26* fragment was amplified from wild-type *Sch. pombe* genomic DNA and subcloned into pGEM-T Easy. *met26* fragments were released by digestion with *NotI* (pGEM-T Easy contains two *NotI* sites flanking the TA cloning site). *met26* was amplified using a newly designed upstream primer *met26S2* and the downstream primer *met26AS* was used to disrupt *met26* (Table 3). pREP41-CYS4 and pREP4H-CYS3 were constructed by integrating full-length CYS4 or CYS3 into the *NdeI*/*BamHI* site of MCS of

pREP41 (Basi *et al.*, 1993) or pREP4H. pREP4H was constructed by replacing the *LEU2* marker region of pREP41 with *his2* as described by Fujita *et al.* (2005). Full-length CYS4 or CYS3 fragments were amplified from wild-type *Sac. cerevisiae* genomic DNA using an upstream primer carrying an *NdeI* site and a downstream primer carrying a *BamHI* site (Table 3).

Homocysteine analysis. The total homocysteine content of each strain was determined using the homocysteine HPLC kit manufactured by Immundiagnostik. Briefly, cells were grown to mid-exponential phase ($OD_{600}=0.8$ – 1.8) in YES or defined MM medium and were collected by centrifugation. Collected cells were washed with deionized water, freeze-dried and 20 mg of each strain was suspended in 600 μ l 8 mM HCl in a microfuge tube and disrupted by vortexing after addition of 0.43–0.6 μ m diameter glass beads (five 30 s pulses). After centrifugation, the supernatant was collected and an aliquot was subjected to fluorescence labelling. HPLC separation was performed on a TSK-GEL ODS-80Ts 4.6 (i.d.) $\times$ 250 mm column (Tosoh). The mobile phase (1 ml min^{−1}) was 2 % methanol containing 0.1 M acetic acid (pH 4.0) and fluorescence was detected using excitation and emission wavelengths of 385 and 515 nm, respectively. The homocysteine assay was performed on two independent samples and the results from only the first assay are shown because the values from the two experiments were nearly the same.

Table 3. Primers used in this study

Primer*	Sequence (5'–3')†
<i>met26S1</i>	GCACACAATTCTTCTCGTCAACTTGAGGC
<i>met26S2</i>	CTACTCACTTTATCACGAGATCTCTCAGCG
<i>met26AS</i>	CGGTAAACCATGTTGAAGGTTAACAACCCC
<i>met14S</i>	GACTCCTAACGAGACGCTAACCGATAGGCG
<i>met14AS</i>	GGGAGCCTCATAGGGAGCAGAAATTCCTG
<i>met16S</i>	TCTGACTATTCTCTAAAAGTCTTACAGAGC
<i>met16AS</i>	GTGCGAATTTACTTTGAGGATGACTGTG
<i>met6up-S</i>	gttttctagaGAACACTACAGGTTTGGCTGGG
<i>met6down-S</i>	gtttaagctAGTATCCTACGGACCAACGG
<i>met6down-AS</i>	gtttctcgagATGTTGGAGGGGTGAGAATG
CYS4SNde	gtttcatatgACTAAATCTGAGCAGCAAGC
CYS4ASBam	gtttggatccGCGTGGGTTCTTATTTATGC
CYS3SNde	gtttcatatgACTCTACAAGAATCTGAT
CYS3ASBam	gttttggatccTGCGGATTAGTTGGTGGC

*S, Sense; AS, antisense.

†Lowercase letters at the start of primer sequences represent restriction enzyme sites.

RESULTS

Sch. pombe met26 mutant exhibits methionine auxotrophy and requires adenine for growth

A search of the Sanger Centre Fission Yeast Genome Sequencing Project (www.sanger.ac.uk/Projects/S_pombe) for an *Sch. pombe* homologue of the *Sac. cerevisiae* methionine synthase gene *MET6* yielded SPAC9.09, designated *met26* as '*met6*' has already been assigned to SPBC56F2.11, encoding homoserine *O*-acetyltransferase. The deduced amino acid sequence of *met26* shared 58% identity with that of *Sac. cerevisiae* Met6p. A *met26* disruptant strain was generated and its growth phenotype was examined. As expected, the *met26* disruptant exhibited methionine auxotrophy, suggesting that *met26* functions in the methionine biosynthetic pathway (Fig. 2). As our original intent was to use *met26* as a new auxotrophic marker for genetic manipulation of *Sch. pombe*, a host strain, YF041, carrying six markers (Table 1) was generated. Unexpectedly, overexpression of *ade7* in this strain could not fully complement the adenine auxotrophy (data not shown). Subsequently, every strain that carried a *met26* mutation was found to barely grow on adenine-free medium. These purine requirements were complemented by overexpression of full-length *met26*, suggesting that the phenotype was caused by the *met26* mutation (Fig. 2).

Other *Sch. pombe* methionine auxotrophs and the *Sac. cerevisiae met6* mutant do not exhibit purine requirement

Other *Sch. pombe* methionine auxotrophs were generated *de novo* to determine if the purine requirement of the *met26* mutant was a generic phenotype of methionine auxotrophy in *Sch. pombe*. *met6*, which has been reported to encode homoserine *O*-acetyltransferase (EC2.3.1.31; Fig. 1, c) (Schweingruber *et al.*, 1998) was disrupted as described in Methods. Homologues of *Sac. cerevisiae* *MET14*, encoding adenylylsulfate kinase (EC2.7.1.25; Fig. 1, a), and *MET16*, encoding 3'-phosphoadenylylsulfate reductase (EC1.8.4.8; Fig. 1, b) were found by searching the *Sch. pombe* genomic

database, yielding SPAC1782.11, designated *met14*, and SPAC13G7.06, designated *met16*. The deduced amino acid sequences of *met14* and *met16* exhibit 65 and 55% identity with those of *Sac. cerevisiae* Met14p and Met16p, respectively. Disruptants were generated and were confirmed to be methionine auxotrophs, indicating that *met14* and *met16* function in methionine biosynthesis. The *met6*, *met14* and *met16* mutants were then tested for adenine auxotrophy with *met26* serving as a control (Fig. 3). Only the *met26* mutant was found to be an adenine auxotroph. A *Sac. cerevisiae met6* strain lacking a functional methionine synthase was also tested and was found not to exhibit adenine auxotrophy, in contrast to the equivalent *Sch. pombe met26* strain (Fig. 3).

Introduction of the cystathionine pathway into *met26* disruptant restores adenine prototrophy

The *Sac. cerevisiae* cystathionine pathway was introduced into the *Sch. pombe met26* mutant to examine the effect of eliminating intracellular homocysteine accumulation. *Sac. cerevisiae* *CYS4*, encoding cystathionine β -synthase (EC4.2.1.22; Fig. 1A, e) and *CYS3*, encoding cystathionine γ -lyase (EC4.4.1.1; Fig. 1A, f), were overexpressed in the *Sch. pombe met26* mutant under the control of the *nmt1* (no message in thiamine) promoter (Basi *et al.*, 1993). Strain YF031 (Table 1) was used as a host for this experiment because an additional selective marker was needed. As a preliminary experiment, *Sac. cerevisiae* Cys4p and Cys3p were tested for activity in *Sch. pombe*. *CYS4* and *CYS3* were co-expressed in the *Sch. pombe* cysteine auxotroph Δ *cys1a*, which lacks the *O*-acetylserine pathway (Fujita & Takegawa, 2004). Co-expression of *CYS4* and *CYS3* in the *cys1a* mutant restored cysteine prototrophy, indicating functional expression in this strain (data not shown). Strain YF031 co-expressing *CYS4* and *CYS3* no longer exhibited a purine requirement, but was still auxotrophic for methionine (Fig. 4, lower row). As expected, the purine requirement persisted in the presence of 5 μ g thiamine ml⁻¹, which suppressed *nmt1*-promoter-driven *CYS4* and *CYS3* expression (Fig. 4, upper row).

Fig. 2. *met26* mutant exhibits methionine and adenine auxotrophy. Identical volumes of 10-fold serial dilutions of the exponentially growing parent strain ARC039, Δ *met26*, *met26*-overexpressed Δ *met26* and empty pAL-transformed Δ *met26* cells were spotted onto MM plates and incubated at 30 °C for 72 h. Basal MM medium was supplemented with 75 μ g adenine (Ade) and/or methionine (Met) ml⁻¹ as indicated under the panels.

Fig. 3. Growth phenotypes of *Sch. pombe* *met* mutants and an *Sac. cerevisiae* *met6* strain. Identical volumes of 10-fold serial dilutions of the exponentially growing parent strain ARC039, $\Delta met14$, $\Delta met16$, $\Delta met6$, $\Delta met26$, *Sac. cerevisiae* parent strain BY4742 and *met6* cells were spotted onto MM or SD plates and incubated at 30 °C for 72 h. Basal MM/SD medium was supplemented with 75 or 100 μg adenine (Ade) and/or methionine (Met) ml^{-1} as indicated under the panels.

Homocysteine accumulates in the *met26* mutant

The above results suggested that the purine requirement of the *met26* mutant was due to intracellular accumulation of homocysteine. Therefore, total intracellular homocysteine was determined. As expected, the amount of total homocysteine in the *met26* mutant was about 18 times higher [62.84 μg (g dry wt) $^{-1}$] than in the parent ARC039 [3.41 μg (g dry wt) $^{-1}$]. Furthermore, total homocysteine in YF031 expressing *CYS4* and *CYS3* was 2.7-fold lower than that in

the empty vector control [9.52 versus 26.11 μg (g dry wt) $^{-1}$; Table 4]. These results demonstrate that the *met26* mutant indeed accumulates a high level of homocysteine.

The *met26* mutation inhibits accumulation of red pigment in *ade7* mutant under adenine-limiting conditions

It has been reported that some purine biosynthetic mutants of yeast accumulate an intensely red pigment when grown

Fig. 4. Introduction of *Sac. cerevisiae* cystathionine pathway restores adenine prototrophy to the *Sch. pombe* *met26* mutant. Identical volumes of 10-fold serial dilutions of exponentially growing YF031, empty vector-transformed YF031, and *Sac. cerevisiae* *CYS4*- and *CYS3*-co-expressed YF031 cells were spotted onto MM plates containing the nutrients indicated under each panel in the presence or absence of 5 μg thiamine (Thi) ml^{-1} and incubated at 30 °C for 7 days. Basal MM medium was supplemented with 75 μg adenine (Ade) and/or methionine (Met) ml^{-1} as indicated under the panels.

Table 4. Total homocysteine levels in methionine auxotrophs

Strain	Homocysteine content [$\mu\text{g (g dry wt)}^{-1}$]
ARC039	3.41
$\Delta met26$	62.84
$\Delta met14$	4.09
$\Delta met16$	4.13
$\Delta met6$	ND*
$\Delta ade7$	3.15
$\Delta met26$ +vectors	26.11
$\Delta met26$ +CYS4, CYS3	9.52

*A level in $\Delta met6$ was not detectable.

in adenine-limiting medium (Roman, 1956; Klein & Favreau, 1988; Zonneveld & van der Zanden, 1995). In *Sch. pombe*, defects in *ade6* and *ade7* lead to accumulation of the intermediate phosphoribosylaminoimidazole (AIR) and phosphoribosylaminoimidazole carboxylate (CAIR), respectively (see Fig. 5a). These intermediates form a characteristic red pigment after transport into the vacuole through the action of a glutathione conjugate pump (Chaudhuri *et al.*, 1996). Parental strain ARC039, the *met26* mutant and the *met26 ade7* double mutant (YF041) did not form a red colour when grown in YE medium, whereas the *ade7* mutant did (Fig. 5b). This result indicates that the *met26* mutation inhibits red pigment formation of the *ade7* mutant under adenine-limiting conditions.

DISCUSSION

Our data indicate that a block in the last step of methionine biosynthesis (catalysed by methionine synthase, Fig. 1, d) not only causes methionine auxotrophy, but also blocks purine biosynthesis in *Sch. pombe*. In this study, *met26* was identified as the *Sch. pombe* methionine synthase gene. A *met26* mutant exhibited a distinct growth defect on adenine-free medium, suggesting a defect in *de novo* purine biosynthesis (Fig. 2). A purine requirement was not observed in other *Sch. pombe* methionine auxotrophs tested, i.e. *met14*, *met16* and *met6* mutants. Furthermore, a purine requirement was not observed in a *Sac. cerevisiae met6* mutant, lacking a functional methionine synthase (Fig. 3). These results suggested the possibility that the defect in purine biosynthesis in the *met26* mutant was caused by intracellular accumulation of homocysteine. In *Sch. pombe*, intracellular accumulation of homocysteine would result from loss of methionine synthase as methionine synthase is the sole enzyme which converts homocysteine into another metabolite, whereas an alternative homocysteine-consuming pathway exists in *Sac. cerevisiae* (Brzywczy *et al.*, 2002). This speculation was strongly supported by the observation that introduction of the *Sac. cerevisiae* cystathionine pathway (Fig. 1A, e–f) into the *Sch. pombe met26* mutant complemented the growth defect on adenine-free medium (Fig. 4). $\Delta met26$ cells co-expressing *Sac. cerevisiae* CYS4 and CYS3 were able to grow on the adenine-free medium, although their growth rate was considerably lower than that of the parent strain ARC039. The lower growth rate of the

Fig. 5. The *met26* mutation inhibits accumulation of red pigment in *ade7* mutant under adenine-limiting conditions. (a) The *de novo* purine biosynthetic pathway and reactions of the conversion and salvage pathways. Intermediates that contribute to the formation of red pigment are highlighted in red. Reactions of a portion of the purine salvage pathway are indicated with broken lines. PRPP, 5'-Phosphoribosyl diphosphate; GAR, 5'-phosphoribosylglycinamide; N-formyl-GAR, 5'-phosphoribosyl-N-formylglycinamide; FGAM, 5'-phosphoribosylformylglycinamide; AIR, 5'-phosphoribosyl-5-aminoimidazole; CAIR, 5'-phosphoribosyl-5-aminoimidazole-4-carboxylate; SAICAR, 5'-phosphoribosyl-5-aminoimidazole-4-N-succinocarboxamide; AICAR, 5'-phosphoribosyl-5-aminoimidazole-4-carboxamide; FAICAR, 5'-phosphoribosyl-5-formaminoimidazole-4-carboxamide; IMP, inosine 5'-monophosphate; AMP, adenosine 5'-monophosphate; Ado, adenosine; XMP, xanthosine 5'-monophosphate; GMP, guanosine 5'-monophosphate. (b) ARC039, $\Delta ade7$, $\Delta met26$ and YF041 ($\Delta met26 \Delta ade7$) strains were grown in YE medium (0.5% yeast extract, 3% glucose) at 30 °C for 48 h.

CYS3- and CYS4-co-expressing $\Delta met26$ strain may be due to the resultant overproduction of cysteine. High levels of cysteine have been reported to be inhibitory to a variety of micro-organisms (Maw, 1961; Allen & Hussey, 1971; Kari *et al.*, 1971).

Measurement of total homocysteine indicated that intracellular levels in the *met26* mutant were higher than in the parent or in the other methionine auxotrophs (Table 4). These data demonstrate that high levels of homocysteine impair purine biosynthesis via direct or indirect mechanisms. In mammals, homocysteine has been reported to induce vascular disease via disturbance of various biological processes, and excessive levels in plasma are closely correlated with cardiovascular disease (Aguilar *et al.*, 2004). Homocysteine induces endothelial dysfunction through an inhibition of lysyl oxidase (LOX) and repression of LOX expression (Raposo *et al.*, 2004). Topal *et al.* (2004) reported that homocysteine induced oxidative stress, which causes vascular damage by inactivating endothelial NO synthase through a reduction of the cofactor tetrahydrobiopterin in human endothelial cells. Homocysteine also diminishes prostacyclin production through inhibition of cyclooxygenase activity (Quere *et al.*, 1995). In *Sac. cerevisiae*, homocysteine has been reported to interfere with ergosterol biosynthesis (Hatanaka *et al.*, 1974; Parks & Casey, 1995). The methionine biosynthetic pathway is linked indirectly to purine biosynthesis via metabolism of folic acid (Fig. 5a), suggesting a metabolic interaction between the methionine and purine biosynthetic pathways. Holmes & Appling (2002) reported that intracellular accumulation of the purine intermediate 5'-phosphoribosyl-5-aminoimidazole-4-carboxamide (AICAR) interferes with methionine biosynthesis in *Sac. cerevisiae*. Therefore, a negative feedback loop between the methionine and purine biosynthetic pathways involving the inhibitory effect of homocysteine may exist in *Sch. pombe*.

While the present study demonstrates an inhibition of purine biosynthesis by elevated homocysteine, the specific target of this inhibition remains unknown. Preliminary analysis using a fission yeast DNA microarray indicated no repression of purine biosynthetic gene expression in *met26* mutant cells, consistent with inhibition occurring at the post-translational level (data not shown). The observation that the *met26* mutation inhibits accumulation of red pigment in $\Delta ade7$ cells under adenine-limiting conditions indicates that homocysteine accumulation interferes with a step prior to the series of reactions catalysed by the enzymes encoded by *ade6* and *ade7* (Fig. 5b). Further work to determine the target for homocysteine inhibition is in progress.

We also tested whether homocysteine accumulation can block purine biosynthesis in *Sac. cerevisiae*. A *met6 cys4* double mutant unable to metabolize homocysteine (see Fig. 1A) was generated and tested for auxotrophies. Whereas the *Sac. cerevisiae met6cys4* double mutant was not an adenine auxotroph, it did exhibit methionine and cysteine auxotrophy (data not shown). Intriguingly,

intracellular accumulation of homocysteine was not observed in the *met6cys4* double mutant when grown in adenine-free SD medium, but a remarkable accumulation of homocysteine was observed when grown in YPD medium (data not shown). It was therefore unclear whether homocysteine accumulation interferes with purine biosynthesis in *Sac. cerevisiae*. Further investigation is needed to elucidate the mechanism of suppression of homocysteine accumulation in the *met6cys4* double mutant grown in adenine-free medium.

To our knowledge, this study describes the first case of inhibition of purine biosynthesis by excessive levels of homocysteine in eukaryotic cells. Homocysteine accumulation in a methionine synthase mutant was found to occur specifically in *Sch. pombe* that possesses a variant pathway of sulfur metabolism. Elucidation of the mechanisms responsible for the homocysteine-inducible defect in purine biosynthesis in *Sch. pombe* may shed light on the mode of action of homocysteine and related metabolites in higher eukaryotes.

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