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Metabolism of Sulfur Amino Acids in *Saccharomyces cerevisiae*

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INTRODUCTION

For all microorganisms, the biosynthesis of sulfur amino acids first requires the capacity to accumulate sulfur atoms from the growth medium and then the transformation of the transported ligands into the reduced form of the sulfur atom, sulfide (S^{2-}). *Saccharomyces cerevisiae* has evolved a large number of both transport and biochemical activities that permit it to use a wide variety of inorganic as well as organic sulfur sources.

The element sulfur occurs in a variety of stable compounds in which its oxidative state can range from −2 in its most reduced form (sulfide) to +6 in its most oxidized form (sulfate). All of these compounds are part of the global sulfur cycle. The available forms of inorganic sulfur atoms in the lithosphere are mainly sulfate and sulfide. In the earth, sulfur is found primarily as sulfate minerals, such as gypsum ($CaSO_4$), or sulfide minerals, such as pyrite (FeS_2). Oceans are the main reserve of sulfur, which is present essentially in the form of inorganic sulfate. Other, less abundant forms of inorganic sulfur, such as thiosulfates, dithionates, polythionates, and elemental sulfur, are also found in the lithosphere. There is a global sulfur cycle resulting from the continuous exchanges of sulfur atoms between the lithosphere and the atmosphere: volatile sulfur gases (sulfur dioxide [SO_2], mainly, and hydrogen sulfide [H_2S]) are emitted to the atmosphere as a result of volcanic activity, decomposition of biological tissues, and artificial human activities. Atmospheric hydrogen sulfide and sulfur dioxide are rapidly oxidized to sulfate, which is deposited on land by rain and in oceans through gaseous deposit (108).

Microbial metabolism of sulfur compounds has led to the formation of a biological sulfur cycle, which constitutes a major part of the global sulfur cycle (Fig. 1). This biological cycle is complex because of the multiple oxidation states of sulfur (Fig. 2). As shown in Fig. 1, microorganisms use the sulfur atom for biosyntheses, as a terminal electron acceptor in a respiratory system, or as an energy source through sulfide or elemental sulfur oxidation. For this review, it is important to note the difference between sulfate assimilatory reduction, where sulfate is taken up and used for the biosynthesis of organic compounds, and sulfate dissimilatory reduction, where the sulfate molecule is reduced as part of a respiratory pathway to sulfite or sulfide, which is not metabolized but excreted. Most eucaryotic microorganisms are able to perform assimilatory reduction of sulfate, whereas, to our knowledge, dissimilatory reduction of sulfate by these organisms has never been reported.

In yeast, as in other sulfate-reducing microorganisms, reduced sulfate is used in the synthesis of organic sulfur metabolites, mostly cysteine, methionine, and *S*-adenosylmethionine (AdoMet). The goal of this review has been to assemble the literature concerning the biosynthesis of sulfur amino acids in *S. cerevisiae*. The biosynthesis of cysteine and methionine in enterobacteria has been the subject of fairly recent reviews (for example, see references 128 and 191).

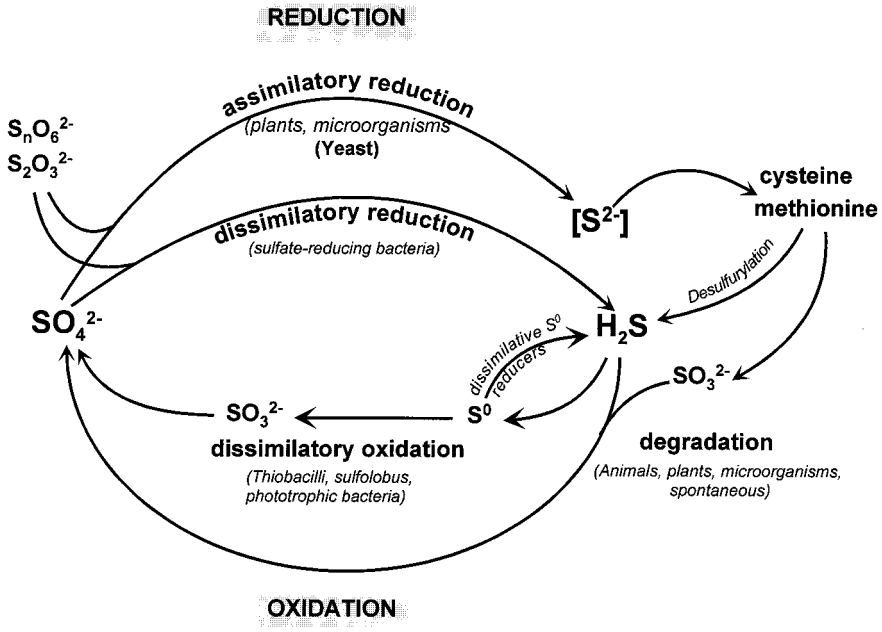

FIG. 1. Biological sulfur cycle.

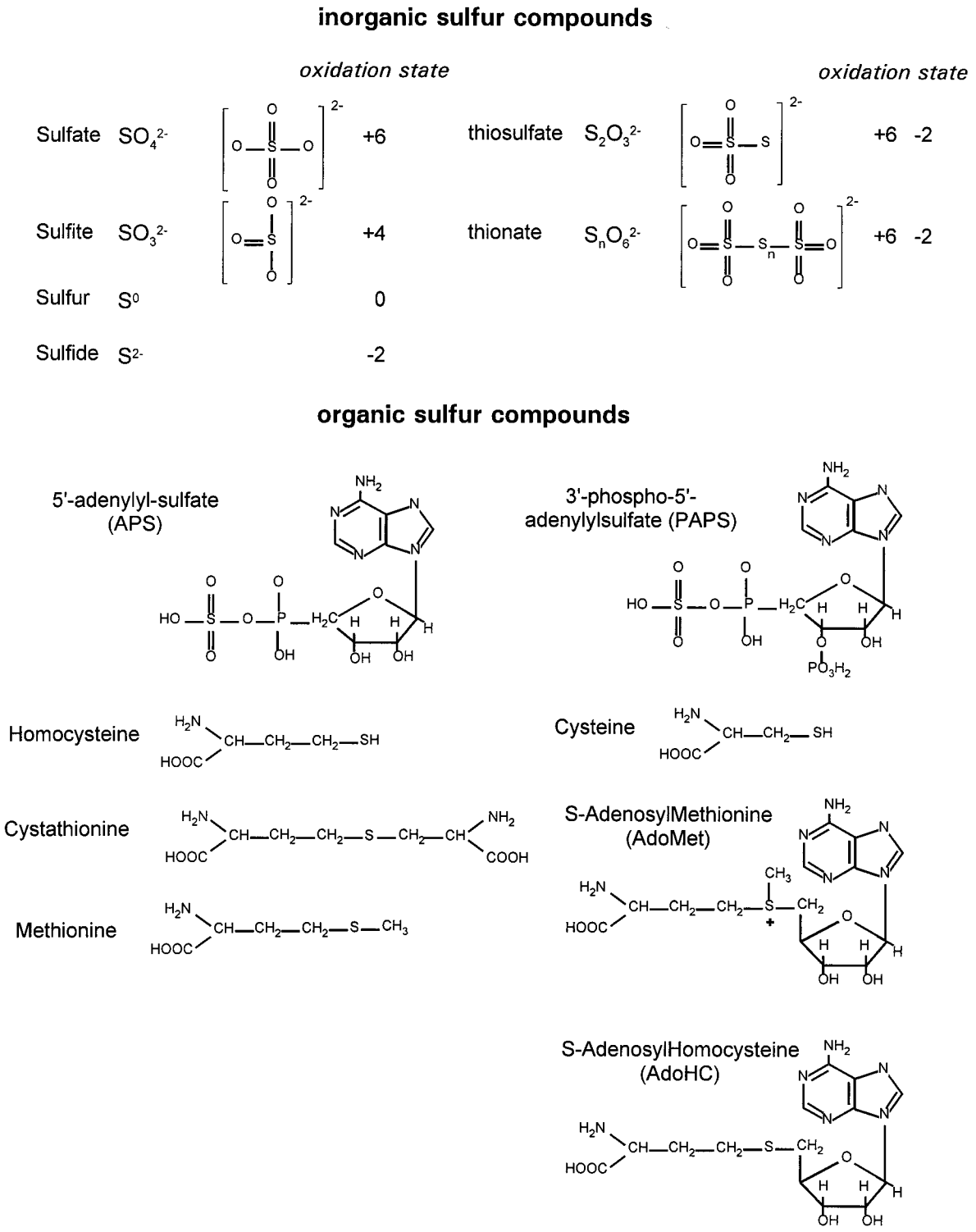

FIG. 2. Inorganic and organic sulfur compounds. The different inorganic sulfur compounds are represented with their oxidation state. The intermediary metabolites and sulfur amino acids are also shown.

A number of excellent reviews of amino acid metabolism and regulation in *S. cerevisiae* are available (94, 107). Therefore, we review here the body of knowledge on the structural genes required for the biosynthesis of sulfur amino acids in *S. cerevisiae* and on the mechanisms underlying the regulation of their expression. In addition, the study of some mutants revealed that seemingly unrelated biological systems are required for the biosynthesis of sulfur amino acids. This review also illustrates how genetic analysis has been essential in the development of our understanding of sulfur metabolism.

TABLE 1. Sulfur amino acid biosynthesis: structural genes

Gene	Alternate name(s)	Chromosome	Enzyme produced	Mutant phenotype
Sulfate assimilation pathway				
MET3	*YJR010w*	X	ATP sulfurylase	Organic sulfur auxotroph[a], resistant to selenate
MET14	*YKL001c*	XI	APS kinase	Organic sulfur auxotroph, resistant to selenate
MET16	*YPR167c*	XVI	PAPS reductase	Organic sulfur auxotroph, resistant to selenate
MET22	*HAL2*, *YOL064c*	XV	Diphosphonucleoside phosphohydrolase	Organic sulfur auxotroph, salt sensitive
MET10	*YFR030w*	VI	Sulfite reductase (α subunit)	Organic sulfur auxotroph
MET5	*YJR137c*	X	Sulfite reductase (β subunit)	Organic sulfur auxotroph
MET1	*MET20*, *YKR069w*	XI	Uroporphyrinogen III methylase	Organic sulfur auxotroph
MET8	*YBR213w*	II	Siroheme synthase (oxidation and chelation)	Organic sulfur auxotroph
Sulfide incorporation and transsulfuration pathways				
MET2	*YNL277w*	XIV	Homoserine transacetylase	*O*-Acetyl homoserine[b] auxotroph Methyl mercury resistant[c]
MET25	*MET17*, *MET15*, *YLR303w*	XII	*O*-Acetylhomoserine sulfhydrylase	Organic sulfur auxotroph, methyl mercury resistant[c]
STR4	*CYS4*, *NHS5, YGR155w*	VII	Cystathionine-β-synthase	Cysteine auxotroph
STR1	*CYS3*, *YAL012w*	I	Cystathionine-γ-lyase	Cysteine auxotroph
Methionine and AdoMet biosynthesis				
MET6	*MET24*, *YER091c*	V	N^5-methyltetrahydrofolate homocysteine transferase	Methionine auxotroph
MET7	*MET23*, *YOR241w*	XV	Tetrahydrofolyl polyglutamate synthetase	Methionine auxotroph
MET13	*YGL125w*	VII	Methylene tetrahydrofolate reductase	Methionine auxotroph
SAM1	*ETH10*, *YLR180w*	XII	AdoMet synthetase	Ethionine resistant
SAM2	*ETH2*, *YDR502c*	IV	AdoMet synthetase	Ethionine resistant, *sam1 sam2* mutant is AdoMet auxotroph

[a] Organic sulfur auxotrophs are able to grow on homocysteine, cysteine, methionine, and AdoMet.
[b] *met2* mutants are also able to grow on all organic sulfur sources except cysteine (see the text).
[c] See reference 233.

The *S. cerevisiae* genes involved in the biosynthesis of sulfur amino acid are listed in Table 1.

SULFUR SOURCES

Growth of *S. cerevisiae* on Inorganic Sulfur Sources

Extensive growth data have been accumulated showing that *S. cerevisiae* possesses various enzymatic systems that permit it to metabolize almost all inorganic sulfur compounds found in the lithosphere. This review will focus on the reductive metabolism of sulfate, which is the best characterized of these systems and is the classical metabolic state of yeast. *S. cerevisiae* is also capable of using the sulfane and sulfonyl sulfur atoms of thiosulfate as well as taking up and metabolizing both sulfite and sulfide. It was also shown that *S. cerevisiae* is also capable of utilizing polythionates and elemental sulfur as sole sulfur sources (14).

Growth of *S. cerevisiae* on Organic Sulfur Sources

In contrast to the bacterium *Escherichia coli* or other fungi such as *Aspergillus nidulans* or *Neurospora crassa* that can use only cysteine, *S. cerevisiae* can grow in the presence of either methionine or cysteine as the sole sulfur source. This unique property results mainly from the fact that yeast cells possess two active transsulfuration pathways catalyzing the interconversion of homocysteine and cysteine. As a consequence, almost all of the structural genes encoding components of sulfur amino acid metabolism were first identified as mutations leading to methionine auxotrophy and therefore were called *MET* genes. In contrast, the structural genes of the sulfate assimilation pathway are known as *CYS* genes in *E. coli*, which cannot use methionine as the sole sulfur source. In yeast, the only mutants that grow on cysteine but not methionine or sulfate are those bearing lesions within the genes encoding the two enzymes catalyzing the homocysteine-to-cysteine transsulfuration pathway. In addition, *S. cerevisiae* is one of the few microorganisms known to transport AdoMet from the medium due to a specific transport system. It has also been shown that *S. cerevisiae* is capable of utilizing aliphatic sulfonates as sole sulfur sources and that sulfate is not an obligatory intermediate in the utilization of these metabolites (256).

The growth characteristics of the different mutant cells, cited in this review, are summarized in Table 2.

SULFATE ASSIMILATION PATHWAY

To assimilate sulfate, all the organisms have been faced with a seemingly complicated electro-redox state. Indeed, SO_4^{2-} is a fairly stable ion, with a low E'_0 for the SO_4^{2-}/SO_3^{2-} redox

TABLE 2. Sulfur nutritional requirements of *MET* mutants[a]

Mutation	Requirement for:					
	Sulfate	Sulfite	Homocysteine	Cysteine	Methionine	AdoMet
None (wild type)	+	+	+	+	+	+
Structural mutants						
met3	−	+	+	+	+	+
met14	−	+	+	+	+	+
met16	−	+	+	+	+	+
met5	−	−	+	+	+	+
met10	−	−	+	+	+	+
met1	−	−	+	+	+	+
met8	−	−	+	+	+	+
met22	−	−	+	+	+	+
met2	−	−	+	−	+	+
met25	−	−	+	+	+	+
str1	−	−	−	+	−	−
str4	−	−	−	+	−	−
met6	−	−	−	−	+	+
met7	−	−	−	−	+	+
met13	−	−	−	−	+	+
sam1, *sam2*	−	−	−	−	−	+
sul1, *sul2*	−	?	+	?	+	+
Regulatory mutants						
met4	−	−	+	−	+	+
met28	−	−	+	+	+	+
cbf1	−	−	+	−	+	+
met30	+	+	+	+	+	+
met31, *met32*	−	−	−	−	+	+
sul3	+	+	+	+	+	+

[a] Unpublished results from our laboratory.

couple (−480 mV), compared to that of NADP/NADPH, the universal reducing fuel molecule, which is about −320 mV. Direct reduction of sulfate into sulfite using reducing equivalents produced by the oxidation of NADPH would therefore be an endergonic reaction (+7.4 kcal/mol). To circumvent this problem, all sulfate-assimilating organisms rely on the activation of sulfate anions into adenylate compounds. Adenylation lowers the electropotential of sulfate so that its subsequent reduction into sulfite and sulfide by means of NAPDH oxidation is feasible (60, 231).

In yeast, sulfate activation is carried out in two sequential reactions: the first transfers the adenosyl-phosphoryl moiety of ATP to sulfate, yielding adenylyl sulfate (APS), which is in turn phosphorylated to yield phosphoadenylyl sulfate (PAPS). The enzymes catalyzing these two reactions are ATP sulfurylase and APS kinase, respectively. For cysteine and methionine biosynthesis, activated sulfate is sequentially reduced to sulfite, which is in turn further reduced to sulfide by sulfite reductase. At the end of this process, the reduced sulfur atom can be incorporated into carbon chains. This set of reactions composes the sulfate assimilation pathway.

Sulfate Activation

Adenylyl sulfate synthesis. Sulfate is activated into APS and pyrophosphate (PP_i) by ATP sulfurylase at the expense of one molecule of ATP (Fig. 3):

$$ATP + SO_4^{2-} \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} APS + PP_i$$

The enzyme from *S. cerevisiae* was first purified to apparent homogeneity by Robbins and Lipmann (213), who pointed out that the equilibrium constant of the reaction is extremely unfavorable for APS synthesis. A few years later, ATP sulfurylase was purified 140-fold and kinetic studies were performed (90). Recently, the kinetic properties of commercial ATP sulfurylase were established in comparison with those of the *Penicillium chrysogenum* enzyme (Table 3) (74).

ATP sulfurylase from *S. cerevisiae* is encoded by *MET3* (39, 40, 177). *met3* mutant cells are unable to use sulfate but can grow on inorganic compounds such as sulfite, sulfide, and thiosulfate, as well as on organic sulfur sources (Table 1). *met3* mutant cells were used to clone (by functional complementation) the yeast *MET3* gene (40) and the cDNA encoding ATP sulfurylase from *Arabidopsis thaliana* (117, 144, 155) and potato (116). The *MET3*-encoded protein has a predicted a molecular weight of 58,000 (Table 3). From results of Robbins and Lipmann (213), Tweedie and Segel calculated that the molecular weight of active ATP sulfurylase was about 100,000 (255). Met3p is thus probably active as a homodimer (Table 3).

The ΔG associated with hydrolysis of the phosphoric/sulfuric acid anhydride bond of APS is quite high, about −19 kcal/mol (153). As a consequence, the apparent equilibrium constant for the reaction catalyzed by ATP sulfurylase is unfavorable (1.1×10^{-8} at pH 8 and 37°C [213]), complicating the acquisition of activated sulfate for metabolism. Several studies dedicated to unraveling how microorganisms have circumvented this problem have been performed.

In *E. coli*, ATP sulfurylase is a heteromeric enzyme, composed of two types of subunits encoded by the *cysN* and *cysD* genes. The native enzyme (390 kDa) is a tetramer of CysDp-CysNp heterodimers (148). The CysNp subunit possesses a GTP hydrolase activity, and the rate of APS synthesis is stimulated by a saturating concentration of GTP (147). Comparison of the primary sequences of CysNp and elongation factor EF-Tu suggests that a common GTP binding motif underlies functional and primary sequence similarities of ATP sulfurylase and other known GTPases (146). Liu et al. further demonstrated that the chemical energy released by CysNp-catalyzed GTP hydrolysis drives APS formation (152, 153).

In *Rhizobium meliloti*, the symbiotic bacterium that stimulates some host legume plants to form nitrogen-fixing nodules, the *nodP* and *nodQ* genes are homologous to the *E. coli cysDNC* region (*E. coli cysC* encodes APS kinase) (61). It has been proposed that NodP and NodQ catalyze the formation of activated sulfate, which is transferred to the nodulation factor. There are two copies of *nodPQ* in the diploid, and only double mutants produce an unsulfated form of the factor (223, 224). There is evidence for a third sulfate activation locus potentially involved in cysteine and methionine synthesis (226). Similar to the CysD protein from *E. coli*, a putative GTP binding site is found in NodQ and GTP is reported to enhance the rate of PAPS synthesis by recombinant NodP and NodQ. Genetic and enzymatic data show that NodQ, in addition to being necessary for ATP sulfurylase activity, has APS kinase activity and is associated with NodP in a multifunctional complex. This suggests that APS formed in the first reaction of sulfate activation may be channeled to the second domain (225) (Fig. 4).

The channeling of activated sulfate is also thought to be of physiological importance in mammalian cells. In rat chondrosarcoma, the ATP sulfurylase and APS kinase activities reside in a single bifunctional protein which uses channeling to efficiently synthesize PAPS from sulfate (157, 158). The mouse cDNA encoding this bifunctional enzyme has been recently cloned, and sequence alignments showed that the enzyme consists of APS kinase and ATP sulfurylase domains separated by a 37-amino-acid linker (149). The importance of metabolite

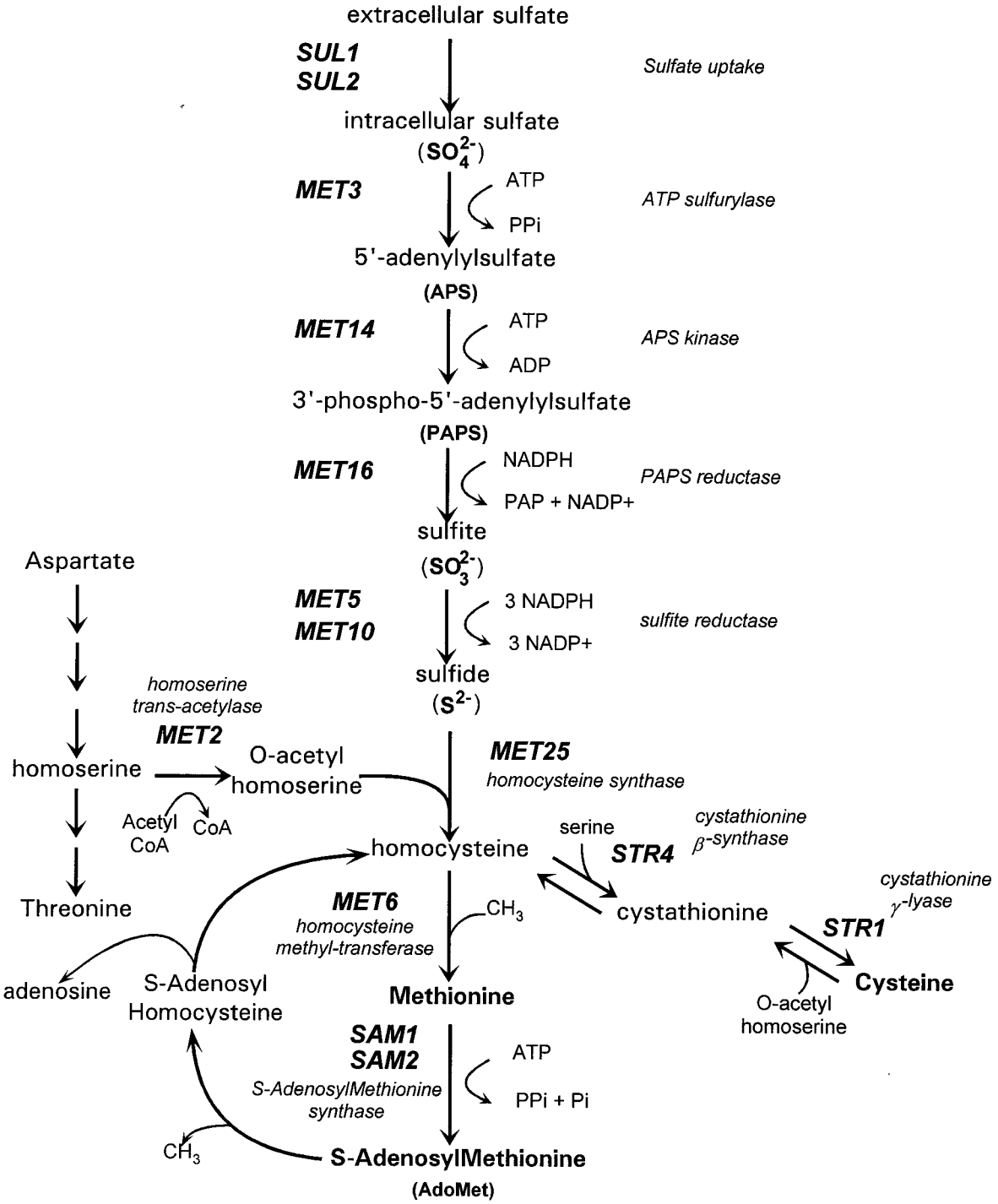

FIG. 3. Sulfur amino acid biosynthesis in *S. cerevisiae.*

channeling by the ATP sulfurylase-APS kinase is emphasized by the observation that a pathologic condition is associated with impairment of this channeling in mice (159). ATP sulfurylase and APS kinase activities are also situated on the same polypeptide in the marine worm *Urechis caupo* (216) (Fig. 4). A related but different enzyme organization was observed in filamentous fungi. However, in these species, fusion of APS kinase and ATP sulfurylase domains does not lead to a bifunctional enzyme but provides an allosteric site. In *P. chrysogenum*, ATP sulfurylase is allosterically inhibited by PAPS. The gene encoding ATP sulfurylase from *P. chrysogenum* has been cloned, and analysis of the sequence showed that the encoded protein is similar to the yeast enzyme over the first 400 amino acids whereas its C-terminal extremity is homologous to the yeast APS kinase. However, this C-terminal region is not endowed with an APS kinase activity but constitutes the allosteric binding site for PAPS (74). The same organization has been found for the *A. nidulans* ATP sulfurylase (18). In both organ-

TABLE 3. Enzyme properties

Enzyme	Assay	K_m (mM)	Mol wt of:		Reference(s)
			Subunit (predicted)	Active structure (experimental)	
ATP sulfurylase	APS synthesis	0.15 (MgATP) 0.95 (SO_4^{2-})	57,800	100,000 (homodimer)	74, 90, 213, 255
	Molybdolysis	0.06 (MgATP) 0.25 (MoO_4^{2-})			
	ATP synthesis	0.56 (APS)			
APS kinase	PAPS synthesis	ND[a]	23,000	49,000–52,000 (homodimer)	222
PAPS reductase	Sulfite synthesis	0.019 (PAPS) 0.0006 (thioredoxin)	30,400	80,000–85,000 (homodimer)	229
Sulfite reductase	Sulfide synthesis	0.017 (sulfite) 0.010 (NADPH)	115,000 (α) 161,000 (β)	604,000 (heterotetramer)$\alpha_2\beta_2$	120–122
Homoserine *O*-transacetylase	*O*-Acetylhomoserine synthesis	1.0 (homoserine) 0.027 (Acetyl-CoA)	53,600	100,000 (homodimer)	266
O-Acetyl homoserine sulfhydrylase	Homocysteine synthesis	ND	48,500	200,000 (homotetramer)	265
Cystathionine β-synthase	Cystathionine synthesis	2.19 (serine) 2.25 (homocysteine)	56,000	235,000 (homotetramer)	195
Cystathionine γ-lyase	Cysteine synthesis	0.25 (cystathionine)	42,400	194,000 (homotetramer)	267
Homocysteine methyltransferase	ND	ND	86,000	ND	
AdoMet synthase I	AdoMet synthesis	0.11 (L-Met) 0.074 (ATP)	41,800	160,000 (tetramer)	47
AdoMet synthase II	AdoMet synthesis	0.14 (L-Met) 0.047 (ATP)	42,200	160,000 (tetramer)	47

[a] ND, not determined.

isms, APS kinase is encoded by another gene and has sequence similarities to Met14p, the APS kinase from *S. cerevisiae* (Fig. 4).

The *S. cerevisiae* Met3p sequence neither shows significant sequence homology to *E. coli* ATP sulfurylase nor possesses an additional functional domain (Fig. 4). However, some observations suggest that channeling of activated sulfate compounds might occur in yeast through protein-protein interactions made between sulfate transporters and enzymes catalyzing sulfate activation and PAPS reduction. It has been observed that strains carrying a mutation in *MET3* (ATP sulfurylase), *MET14* (APS kinase), or *MET16* (PAPS reductase) are all defective in sulfate transport (20, 245), suggesting that each of the corresponding enzymes might interact with the sulfate transporters and therefore that a multicomponent complex might be involved in sulfate transport and activation. It is noteworthy that the transport defect was shown not to be the result of transport inhibition by an intermediary compound of the sulfate assimilation pathway, because lack of sulfate transport was measured with cells growing in the presence of homocysteine as the sole sulfur source. The possibility that a multicomponent complex exists in yeast was further strengthened by analysis of *met3* mutant cells expressing the *ASA1* gene, which encodes ATP sulfurylase from *Arabidopsis thaliana* chloroplasts. In such cells, ATP sulfurylase but not sulfate uptake activity was restored. This result may be accounted for by the inability of the plant enzyme to assemble with yeast sulfate transporters and activating enzymes to form a functional multienzymatic complex (155). However, formal proof of the existence of such a complex is still missing. It cannot be excluded that a functional sulfate-reducing pathway might remove the pathway intermediates, thus displacing the equilibrium of the ATP sulfurylation reaction (228). In addition, hydrolysis of PP_i into phosphate by pyrophosphatase might favor the formation of APS. However, if the intracellular concentration of PP_i in *E. coli*, which has been estimated to be around 0.5 mM, can be extrapolated to

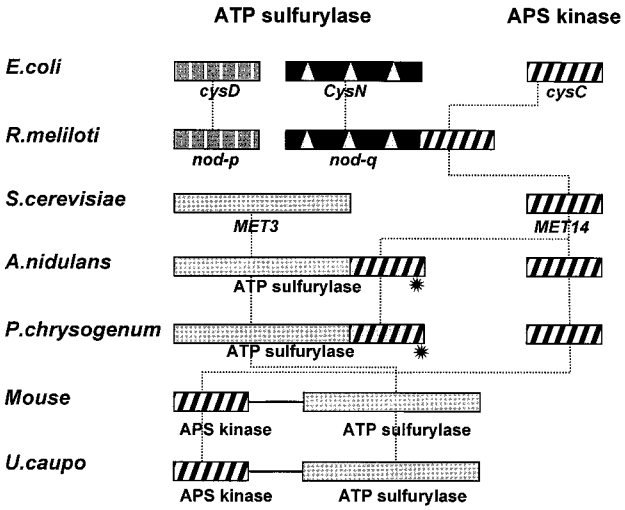

FIG. 4. ATP sulfurylase and APS kinase gene structures and similarities in bacteria, fungi, and higher eukaryotes. Similar regions are shaded appropriately (18, 39, 74, 148, 149, 216, 226). ∗, The APS kinase-like region of ATP sulfurylase of *A. nidulans* and *P. chrysogenum* is not active as a kinase.

yeast cells, this reaction would lie far from equilibrium in vivo and would have no consequences on APS formation (146).

The above-described mechanisms underlying sulfate activation suggest that various metabolic strategies have emerged during evolution from either independent gene fusion events or enzyme recruitment, as in the case of the ATP sulfurylase from *E. coli*. To date, how the sulfate anion is really activated in yeast is not understood.

Role of APS in thermotolerance. Jakubowski and Goldman have observed that *S. cerevisiae* cells grown at 30°C under methionine-repressing conditions (103) lose viability upon transfer to 45°C whereas they survive the transfer in the absence of methionine. This methionine-mediated cell death at high temperature can be explained by the protective effect of intracellular APS. Indeed, APS is elevated after a temperature shift, and cells unable to synthesize this intermediate do not survive the temperature shift. The inability to synthesize APS can arise either from repression of APS synthesis by growth in the presence of methionine or from a *met3* mutation. The authors concluded that methionine-mediated cell death at high temperature is linked to the repression of the synthesis of APS (103).

PAPS synthesis. In yeast, after being activated to APS, the sulfate anion is again phosphorylated, yielding PAPS. PAPS synthesis is catalyzed by APS kinase at the expense of one molecule of ATP (Fig. 3):

$$\text{APS} + \text{ATP} \overset{\text{Mg}^{2+}}{\rightleftharpoons} \text{PAPS} + \text{ADP}$$

APS kinase from *S. cerevisiae* has been purified to homogeneity (222). The molecular weight of the purified enzyme was shown to be about 50,000 by gel filtration and 28,000 by sodium dodecyl sulfate (SDS)-denaturing gel electrophoresis, suggesting that the native enzyme is a homodimer (222) (Table 3). In addition, *S. cerevisiae* APS kinase is activated in vitro by thioredoxin (222). A yeast mutant that does not express functional thioredoxin is now available (76, 179). It will thus be possible to test whether APS kinase activity depends on the presence of thioredoxin in vivo.

In *S. cerevisiae*, APS kinase is encoded by *MET14* (168). The *MET14* gene has been cloned by Fitzgerald-Hayes et al. (70) and sequenced by Korch et al. (125). The *MET14*-encoded product has a molecular weight of 23,000, which correlates with the experimental value found by SDS-polyacrylamide gel electrophoresis (222) (Table 3). Met14p exhibits significant sequence similarities to the APS kinases from bacteria, fungi, and plants (Fig. 4). As described in the preceding section, depending on the organisms, the APS kinase is found either fused to the ATP sulfurylase or not, but all the APS kinase domains are highly related polypeptides.

A futile PAPS-APS cycle in yeast? There is strong evidence that PAPS is an extremely toxic compound, and it seems that organisms have evolved enzymatic systems that permit fine-tuning of the intracellular PAPS concentration (217). These controls could be done by the action of Met22p related proteins. Mutations in *MET22* were originally isolated as methionine auxotrophic mutants (167). Further analysis showed that *met22* mutants could grow on methionine or AdoMet but not on inorganic sulfur (sulfate or sulfite). This phenotype was not understood because in vitro enzymatic studies showed that extracts of *met22* mutant cells contained wild-type levels of all enzymes participating in sulfate assimilation (244, 245); such cells are able to synthesize methionine (253). The *MET22* gene was cloned (Table 2) and shown to be identical to *HAL2*,

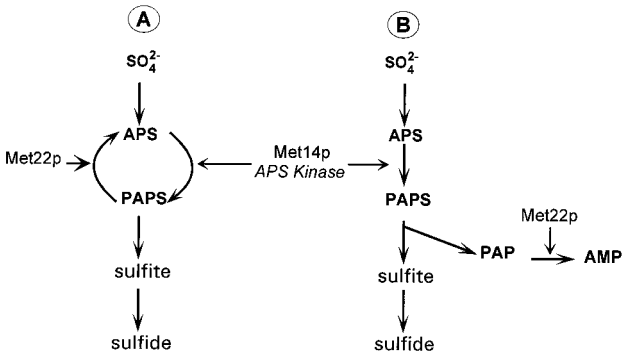

FIG. 5. Met22p function. (A) The APS-PAPS futile cycle (see the text and reference 206). (B) Elimination of PAP from the intracellular pool (181, 182).

whose overexpression improves yeast growth under salt stress (81).

The *MET22* (*HAL2*)-encoded product is a 40-kDa protein that displays sequence similarities to members of a protein family which includes animal inositol phosphatases, the CysQ protein needed for cysteine synthesis in *E. coli*, and several bacterial and fungal proteins of unknown function (187, 188). These observations were taken as an indication that Met22p might be indeed a phosphatase (81). This hypothesis was supported by different observations. Met22p (Hal2p) from yeast was purified and shown to be capable of hydrolyzing, with the same efficiency, both PAPS and phosphoadenosine phosphate (PAP) (Fig. 5) (181; also see below). It was also reported that Met22p activity was inhibited by lithium and sodium ions and that this inhibition was overcome by potassium ions (181). From these results, it was postulated that the cation sensitivity of Met22p (Hal2p) would be an important determinant of the salt sensitivity exhibited by yeast cells, explaining why overexpression of Met22p results in NaCl- and LiCl-resistant cells (181). However, this last hypothesis is questioned by the fact that a chromosomal deletion of *MET22* generates greater LiCl sensitivity than that in the wild-type cells (253). Murguia et al. later postulated that, in vivo, the Met22p substrate would be PAP and not PAPS (Fig. 5B) (182). Different insights into the in vivo function of Met22p were provided by studies of Met22p analogs in plants. Two genes encoding Met22p analogs were isolated from plants, one from rice (*RHL*) (206) and the other from *Arabidopsis thaliana* (*SAL1*) (210). When expressed in yeast, both Rhlp- and Sal1p-encoding cDNAs complement the methionine auxotrophy of *met22* mutant cells, suggesting that the two proteins from plants and Met22p are endowed with similar enzymatic activities. The two plant enzymes were purified, and both catalyzed the hydrolysis of PAPS much more efficiently than that of PAP. The Rhlp and Sal1p enzymes catalyze the hydrolysis of PAPS into APS and were thus referred to as 3′(2′),5′-bisphosphonucleoside 3′(2′)-phosphohydrolase, an enzyme previously described in plants (254). Both Rhlp and Sal1p activities were reported to be strongly inhibited by lithium and sodium ions. Such an enzymatic activity establishes a PAPS-APS futile cycle, whose operation might protect cells against the toxic effect of PAPS (206) (Fig. 5A). The consequence of both models (depicted in Fig. 5A and B) is that the absence of Met22p would result in the accumulation of PAPS, whose toxicity would prevent the mutant cells from growing. *met22* mutants would thus require methionine for growth because methionine would prevent PAPS from accumulating by mediating the repression of uptake and activation of sulfate. Addition of sulfite would not be expected to relieve

the PAPS toxicity, since it does not bring about a repression of the sulfate assimilation pathway. However, a block upstream (in *MET3* for example) prevents the accumulation of PAPS and allows the growth of *met3 met22* double mutants on sulfite (253).

Sal1p from *Arabidopsis thaliana* was shown to be capable of hydrolyzing inositol polyphosphate in vitro, and *SAL1* overexpression in *met22* cells significantly increased lithium and sodium effluxes in addition to restoring methionine prototrophy. This suggests that Sal1p may be a bifunctional enzyme that, apart from its role in the sulfate assimilation pathway, may participate in the phosphoinositide signaling pathway, thus explaining why *SAL1* overexpression protects yeast cells from high salt concentrations (210). The possibility that yeast Met22p is also capable of hydrolyzing inositol phosphates is raised by the fact that *met22* mutant cells exhibit decreased calcium effluxes (253).

Sulfate Reduction

After being activated, sulfate is reduced by two successive steps that finally lead to sulfide formation. The oxidation of four molecules of NADPH is required to perform these reductions.

Sulfite synthesis. The first reduction reaction is catalyzed by PAPS reductase, which yields sulfite from PAPS at the expense of one molecule of NADPH (Fig. 3):

$$PAPS + NADPH \rightarrow PAP + NADP^+ + SO_3^{2-} + H^+$$

As early as 1961, Wilson et al. fractionated the NADPH-PAPS reductase system from yeast into three protein components (3, 262). This system was further studied by Gonzalez Porque et al. (82, 83), who showed that yeast thioredoxin and thioredoxin reductase could substitute for two of the fractions described by Wilson et al. PAPS reductase was purified from yeast and shown to be a homodimer of 80,000 to 85,000 with reduced thioredoxin as the cosubstrate (Table 3). From kinetic data, it was concluded that the enzyme follows an ordered mechanism, binding first thioredoxin and then PAPS (229). The participation of thioredoxin in sulfate reduction was further supported by the isolation and analysis of two genes from *S. cerevisiae* encoding thioredoxins, *TRX1* and *TRX2* (76, 179, 180). Deletion of either *TRX1* or *TRX2* does not affect cell growth and morphology, but simultaneous deletion of both thioredoxin-encoding genes profoundly affects the cell cycle (the S phase is threefold longer, and G_1 is virtually absent) and results in methionine auxotrophy. This result indicates that in contrast to what occurs in *E. coli* (217), the yeast glutaredoxin cannot replace thioredoxin in sulfate reduction.

The *S. cerevisiae* PAPS reductase is encoded by *MET16* (15, 168, 245), and the active enzyme is a dimer (229) (Table 3). Met16p has strong sequence similarities to PAPS reductase from *E. coli*, bacteria, and different fungi. Three different *Arabidopsis thaliana* genes have been cloned by functional complemntation of a *cysH* mutant of *E. coli*, (defective in PAPS reductase). The corresponding proteins were shown to use APS more readily than PAPS as the substrate in vitro, and were thus called APS reductase. They all contain a thioredoxin-like domain that may be involved in the catalytic function, since efficient APS reduction is observed in the absence of added thioredoxin (87). These results support the possibility that sulfate assimilation in plants occurs via direct reduction of APS to sulfite without activation of sulfate to PAPS. However, the presence of a sulfate assimilation pathway involving PAPS as an intermediary metabolite in plants is suggested by the existence of thioredoxin-dependent PAPS reductase activity in spinach extracts (228) as well as by the presence of PAPS hydrolase activity in *Arabidopsis thaliana* and rice (see above).

To prevent detrimental PAPS accumulation in cells devoid of PAPS reductase, different mechanisms can operate. As stated above, in *S. cerevisiae*, cells bearing a chromosomal deletion of *MET16*, as well as the *trx1 trx2* double mutant, do not possess sulfate uptake activity. In *E. coli*, *cysH* mutants (devoid of PAPS reductase), as well as thioredoxin-glutaredoxin double mutants which are unable to reduce PAPS, show limited growth ability unless they accumulate secondary mutations, impairing the first steps of sulfate assimilation, in *cysA* and *cysC*, which encode the sulfate permease and the APS kinase, respectively (77, 217). However, in contrast to what is observed in *E. coli*, the loss of sulfate uptake activity in yeast does not result from inactivation of the sulfate uptake genes but, rather, from a different and reversible mechanism. Indeed, sulfate transport is restored when the mutant cells are transformed by a plasmid bearing the wild-type allele of *MET16*. In filamentous fungi like *P. chrysogenum* or *A. nidulans*, overaccumulation of PAPS seems to be prevented through PAPS-mediated inhibition of the ATP sulfurylase (18, 74) (see above).

Sulfide synthesis. The reduction of sulfite to sulfide occurs at the expense of oxidizing three molecules of NADPH (Fig. 3).

$$SO_3^{2-} + 5H^+ + 3NADPH \rightarrow H_2S + 3H_2O + 3NADP^+$$

Sulfite reductase, which catalyzes the direct six-electron reduction of sulfite into sulfide, has been purified to homogeneity from bacteria and yeast (200, 201, 269). The *S. cerevisiae* enzyme contains different prosthetic groups: a flavin adenine dinucleotide (FAD), a flavin mononucleotide (FMN), an iron-sulfur cluster, and a particular prosthetic group, the siroheme, which is synthesized from uroporphyrinogen III. The yeast sulfite reductase has an $\alpha_2\beta_2$ oligomeric structure with a total molecular mass of 604,000 (Table 3), with the molecular mass of the α and β subunits being of 116,000 and 167,000, respectively. The enzyme contains two FADs, two FMNs, and two sirohemes per active molecule (120–122, 269). The *E. coli* enzyme is somewhat different, having an $\alpha_8\beta_4$ structure, with the molecular weights of the subunits being 66,000 (α subunit, flavoprotein encoded by *cysJ*) and 64,000 (β subunit, hemoprotein encoded by *cysJ*). The bacterial enzyme contains four FADs and four FMNs per active molecule; it is possible to separate the enzyme into functional flavo and hemo components (232). This is not possible for yeast sulfite reductase, which loses all of its prosthetic groups and associated enzymatic activities upon mild denaturation (121).

In *S. cerevisiae*, the α subunit of sulfite reductase is encoded by *MET10*. The *MET10* gene product consists of 1,035 amino acids residues with a predicted molecular weight of 115,000, corroborating biochemical results (120) (Table 3). Met10p and CysJp exhibit low sequence similarities, which are restricted to the carboxy-terminal regions. The β subunit of the *S. cerevisiae* sulfite reductase is encoded by *MET5*, which had been mapped on chromosome X (174) and probably corresponds to the *YJR137c* open reading frame (ORF). Indeed, the polypeptide deduced from the *YJR137c* ORF shows significant similarities to the β subunit of sulfite reductase from *E. coli* and has a predicted molecular weight of 161,000 (Table 3), which correlates with the molecular weight of the sulfite reductase β subunit purified from yeast. The size of the *YJR137c* ORF is, moreover, in accord with the size of the *MET5* transcript (5.5 kbp [176]). Cells bearing a mutation in the *MET5* gene exhibit the same phenotype as *met10* mutants.

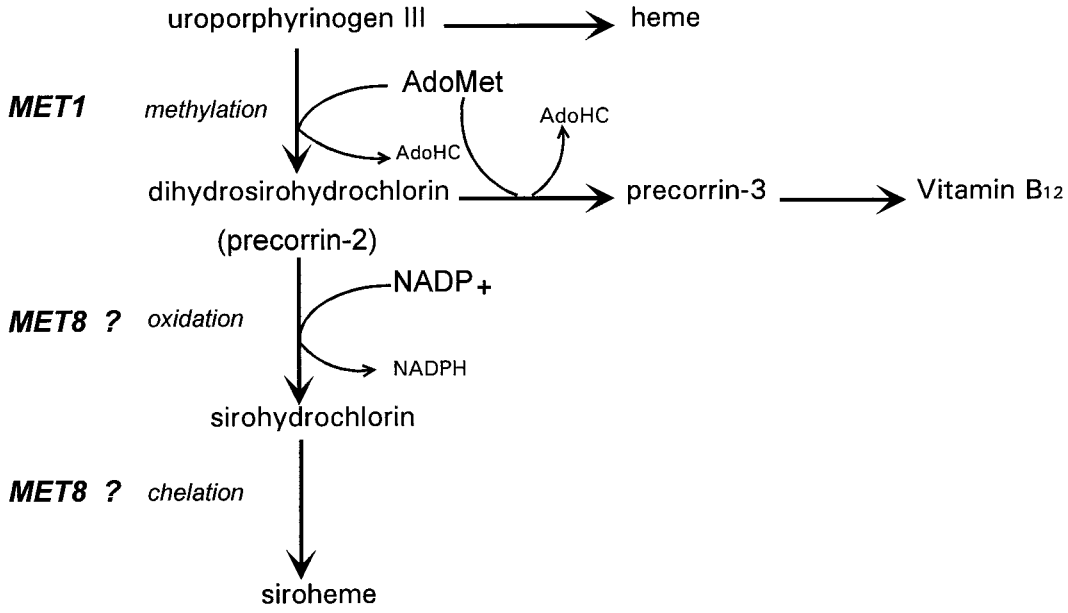

FIG. 6. Siroheme biosynthesis (88, 260).

Siroheme synthesis. Functional sulfite reductase requires the biosynthesis of a particular heme molecule, called siroheme. Only siroheme and siroheme proteins were shown to react with sulfite (230, 270). In *E. coli* and *Salmonella typhimurium*, siroheme is derived from uroporphyrinogen III through two methylations, an oxidation, and a chelation (Fig. 6). In both bacterial species, one enzyme, the siroheme synthetase, catalyzes all three reactions and is encoded by the *cysG* gene. Functional dissection of *E. coli cysG* has shown that a truncated protein containing the carboxy-terminal part of CysG (amino acids 202 to 247) is able to perform the methylation of uroporphyrinogen III as efficiently as the complete *cysG* product does. As expected, a plasmid bearing the corresponding truncated *cysG* gene was unable to complement a *cysG* mutation. The amino-terminal part of CysG has therefore been suggested to contain the oxidation and chelation activities (260).

In *S. cerevisiae*, *MET1* and *MET8* gene products are involved in siroheme synthesis (88). *MET1* (identical to *MET20*) encodes a polypeptide of 593 residues with a molecular weight of 66,000, and *MET8* encodes a polypeptide of 274 residues with a molecular weight of 32,000. The carboxy-terminal part of Met1p shows significant sequence similarities to the carboxy-terminal parts of both the *E. coli* CysGp and CobAp from *Pseudomonas denitrificans*, while the amino-terminal extremity of CysGp has sequence similarities to Met8p. These sequence similarities, as well as the fact that expression of the bacterial CysG protein restores the methionine prototrophy in both *met1* and *met8* yeast mutants, have led to the conclusion that Met1p catalyzes the methylation of uroporphyrinogen III and Met8p catalyzes the oxidation and chelation to yield siroheme (88) (Fig. 6).

Sulfite toxicity. Sulfite is a potentially toxic metabolite in *S. cerevisiae* as well as other microorganisms. It is also a reducing agent. These properties are exploited in the food industry, where sulfite is used as a powerful antioxidative and antimicrobial agent. The molecular basis of sulfite-induced inhibition of growth is still not completely understood, although it has been suggested that sulfite resistance could be acquired through the production of sulfite-binding compounds, such as acetaldehyde (207). Yeast mutants capable of growing in the presence of high sulfite concentrations (1 mM) have been identified; seven mutants were shown to bear dominant mutations in a single gene (32, 33). This gene, *FZF1* (formerly *SUL1*), is predicted to encode a zinc finger protein (31). Acetaldehyde production is significantly higher in *fzf1* mutants than in the wild type (32). Independently, 19 sulfite-resistant mutants were isolated; 11 bear dominant mutations in the *RSU1* locus (264). Whether *RSU1* and *FZF1* are the same gene has not been tested.

In a second approach, mutant cells that are more sensitive to sulfite than are wild-type cells have also been isolated (264). Four complementation groups defining the genes *SSU1* to *SSU4* were identified. None of the sulfite-sensitive mutants were impaired in sulfate assimilation. Decreased acetaldehyde excretion may account for the sensitivity of at least some mutants (*ssu2-6*, *ssu3-7*, and *ssu4-11*) (264). Two mutants (*ssu2-6* and *ssu3-7*), moreover, exhibited cross-sensitivity to other metabolic inhibitors. Further studies showed that *SSU2* is identical to *GRR1*, whose product is a putative global regulatory protein (references 12, 113, and 203 and references therein). Overexpression of the *FZF1* gene from a multicopy plasmid suppressed the sulfite sensitivity of *grr1* mutants but not their other phenotypes. Taken together with the fact that cells containing a chromosomal deletion of *FZF1* are sulfite sensitive, these results raise the possibility that Fzf1p functions as a regulator of sulfite metabolism. Since overexpression of Fzf1p suppresses the sulfite sensitivity of several mutants but not that of a *met1* (*met20*) mutant, Fzf1p could be a positive regulator of *MET1* gene expression. Since Met1p is implicated in the biosynthesis of siroheme, this would suggest that the increase of sulfite reduction could be one route to sulfite detoxification (4).

Sulfite in fermented beverages. Intermediates of the biosynthesis of sulfur amino acids play an important role in the flavor and the conservation of wine and beer. In beer brewing, sulfite stabilizes the flavor by forming adducts with aldehydes whereas sulfide is generally regarded as undesirable. Inadequate amounts

of sulfite are sometimes produced in brewer's yeast; therefore, means of controlling sulfite production have been designed. An increase in sulfite production was obtained by Korch et al. by increasing the gene dosage of *MET3* and *MET14* in *S. cerevisiae* (126). Increased sulfite production in brewer's yeast was obtained by Hansen and Kielland-Brandt by constructing an allotetraploid strain of *Saccharomyces carlsbergensis* and inactivating the four copies of *MET10*. The brewing performance of the resulting strain was satisfactory, as was the taste of the beer obtained by the use of this strain (89).

Liberation of sulfide is the main problem afflicting wine fermentation; therefore, the regulation of hydrogen sulfide liberation in wine-producing *S. cerevisiae* has been studied. It was shown that ammonium and most amino acids added to the growth medium are able to suppress the liberation of excess hydrogen sulfide, suggesting that any compound that can efficiently generate sulfide-binding nitrogenous precursors of organic sulfur compounds can prevent the liberation of excess hydrogen sulfide (105).

Sulfur Incorporation into a Carbon Chain

The last committed step of the sulfate assimilation pathway is catalyzed by Met25p and consists of the incorporation of sulfide into a carbon chain. In *E. coli* and plants, sulfide is incorporated into a three-carbon chain yielding cysteine, which is transformed into homocysteine by the transsulfuration pathway. Homocysteine is the immediate precursor of methionine biosynthesis (78, 127). It was only recently demonstrated for yeast, in contrast to *E. coli* and plants, that sulfide is incorporated into a four-carbon chain, yielding homocysteine. Actually, no direct synthesis of cysteine from sulfide occurs in yeast: cysteine is formed only through the transsulfuration pathway. There is at least one example of a gram-positive bacterium in which the sulfide ion is incorporated into a four-carbon chain and not into a three-carbon chain like in *E. coli*. In *Pseudomonas aeruginosa*, sulfur incorporation occurs only through the sulfhydrylation of *O*-succinylhomoserine in a reaction catalyzed by the product of the *metZ* gene (72).

The biosynthesis of cysteine in *S. cerevisiae* was unraveled by genetic analysis of two cysteine auxotrophs (*str1* and *str4*) that could not grow on methionine or homocysteine (41). *str1* and *str4* mutant cells lack cystathionine γ-lyase and cystathionine β-synthetase activity, respectively (41). Definitive demonstration that conversion of homocysteine into cysteine is the only means of de novo cysteine synthesis in yeast was obtained through the inactivation of both the *STR1* and *STR4* genes, which individually result in cysteine auxotrophic cells (13, 46). *STR1* and *STR4* were shown to be equivalent to *CYS3* and *CYS4*, respectively (193, 197, 198). It was also shown that *CYS1* and *CYS2*, which were thought to also be mutated in *cys3* and *cys4* mutants, respectively, did not exist (197, 198).

***O*-Acetylhomoserine synthesis.** A prerequisite for the biosynthesis of homocysteine is the activation of homoserine through esterification. Depending on the organism, either *O*-succinyl-, *O*-acetyl- or *O*-phosphorylhomoserine is synthesized. Enteric bacteria use *O*-succinylhomoserine (191), while several gram-positive bacteria, yeasts, and fungi use *O*-acetylhomoserine (184, 185, 202, 214, 266). In plants, *O*-phosphorylhomoserine is widely used. Exceptions are *Pisum sativum* and *Lathyrus sitivus*, which synthesize *O*-acetylhomoserine and *O*-oxalylhomoserine, respectively (59, 79).

In *S. cerevisiae*, homoserine *O*-acetyltransferase catalyzes a reaction in which one molecule of acetyl coenzyme A (acetyl-CoA) is consumed (Fig. 3):

$$\text{L-Homoserine} + \text{acetyl-CoA} \rightarrow \textit{O}\text{-acetylhomoserine} + \text{CoA}$$

The same enzyme is also capable of catalyzing the in vitro exchange of the acetyl group between *O*-acetylhomoserine and L-homoserine (111):

$$\text{L-}[^3\text{H}]\text{Homoserine} + \textit{O}\text{-acetylhomoserine} \rightarrow \text{L-homoserine} + \textit{O}\text{-}[^3\text{H}]\text{acetylhomoserine}$$

The *S. cerevisiae* enzyme has been partially purified from a methionine auxotroph, (probably *met10*) impaired in sulfite reductase activity (266). The kinetic parameters have been determined (Table 3), and the molecular weight of the active enzyme has been estimated to be about 100,000, indicating that it is a dimer (Table 3) (266).

In *S. cerevisiae*, homoserine *O*-acetyltransferase is encoded by *MET2* (38). *met2* mutant cells grow on *O*-acetylhomoserine, homocysteine, methionine, or AdoMet as a sulfur source (Table 2) but are unable to grow on cysteine, since *O*-acetylhomoserine is required for the synthesis of cystathionine from cysteine (Fig. 3). For the same reason, a *met4* mutant is unable to grow on cysteine, since Met4p is the transcriptional activator of *MET2* (see below). *met2* mutants have also been isolated as methyl mercury-resistant cells (234). Resistance arises from intracellular accumulation of hydrogen sulfide (194). *MET2* encodes a protein with a predicted molecular weight of 53,000 (11, 140), corroborating the proposed dimer structure of the native enzyme (Table 3). The Met2p sequence is similar to its functional homolog from the fungus *Ascobolus immersus* (84).

Homocysteine synthesis. Homocysteine is the product of the *O*-acetylhomoserine sulfhydrylase reaction (Fig. 3):

$$\textit{O}\text{-Acetylhomoserine} + S^{2-} \rightarrow \text{homocysteine} + H_2O$$

As stated above, this reaction is the only one allowing sulfur incorporation into a carbon chain in yeast. *O*-Acetylhomoserine sulfhydrylase was purified to homogeneity and shown to be a homotetramer with a molecular weight of 200,000 and to bind four molecules of pyridoxal phosphate (Table 3) (265, 268). The amino acid composition of the purified enzyme was also determined and corresponds to that of the deduced *MET25*-encoded product (110, 265). *MET25* is identical to *MET15* identified by Singh and Sherman when they searched for methyl mercury-resistant strains (233, 234). As with *met2* mutants, *met25* cells are methyl mercury resistant because they accumulate hydrogen sulfide, which can be visualized on colonies and serves as a useful color test (54, 244). Met25p has significant sequence similarities to the *E. coli* cystathionine γ-synthase and cystathionine β-lyase (encoded by *metB* and *metC*, respectively) and cystathionine γ-lyase from *S. cerevisiae* and rats. All of these enzymes thus appear to belong to one protein family, whose members have evolved from an ancestral pyridoxal phosphate enzyme (43).

BIOSYNTHESIS OF CYSTEINE AND METHIONINE

Transsulfuration Pathways

Transsulfuration pathways consist of reactions that allow the interconversion of homocysteine and cysteine via the intermediary formation of cystathionine (Table 2). The conversion of cysteine to homocysteine is the only means of transsulfuration in enteric bacteria. In contrast, mammalian cells possess only the homocysteine to cysteine pathway. In *S. cerevisiae*, both transsulfuration pathways exist and involve two different sets of enzymes. The presence of two active transsulfuration pathways in the same organism is not a yeast-specific feature, since it also occurs in several archebacteria species (271).

Synthesis of cysteine from homocysteine. The synthesis of cysteine from homocysteine requires two successive steps, β addition and γ elimination (Fig. 3). Cystathionine β-synthase catalyzes the first reaction:

$$\text{Homocysteine} + \text{serine} \rightarrow \text{cystathionine} + H_2O$$

The enzyme was purified to homogeneity. The molecular mass of the homogeneous enzyme was estimated to be 235,000 by gel filtration and 55,000 by SDS-polyacrylamide gel electrophoresis (Table 3). Amino-terminal sequencing demonstrated that the subunits are identical and that the native enzyme is a homotetramer (195). In *S. cerevisiae*, cystathionine β-synthase is encoded by *STR4* (*CYS4*) (41, 193). As explained in the preceding section, *str4* (*cys4*) mutants are cysteine auxotrophs (Table 2). This nutritional requirement can be also satisfied by the addition of glutathione to the growth medium, since this peptide is efficiently hydrolyzed in vivo to yield cysteine (41, 46). *STR4* encodes a polypeptide of 506 residues, which shows extensive sequence similarity to its functional analog from rats, which contains an additional amino-terminal extension of 60 residues. Moreover, the two enzymes are closely related to the cysteine synthase from enteric bacteria and plants (46). It is noteworthy that all of these proteins catalyze β-replacement reactions. The human cystathionine β-synthase complements the cysteine auxotrophy of a *str4* mutant. This has been used to develop a yeast assay for functional detection of mutations in the human enzyme (131, 132).

Cystathionine γ-lyase catalyses the γ cleavage of cystathionine, the second step of the biosynthesis of cysteine from homocysteine.

$$\text{L-Cystathionine} + H_2O \rightarrow \text{L-cysteine} + \alpha\text{-ketobutyric acid} + \text{ammonia}$$

Cystathionine γ-lyase has been purified to homogeneity and has a molecular weight of about 194,000 (Table 3). Since the subunit has a molecular weight of 48,000, the native enzyme may be homotetramer (267) (Table 3). In *S. cerevisiae*, cystathionine γ-lyase is encoded by *STR1* (*CYS3*), whose mutation leads to a nutritional requirement for cysteine or glutathione (41, 46). *STR1* (*CYS3*) encodes a protein of 393 amino acids with a predicted molecular weight of 42,000, agreeing with that of the purified enzyme (13, 41, 199, 267). As noted above, the yeast cystathionine γ-lyase belongs to a protein family which includes its functional analog from rats, Met25p from yeast, and cystathionine β-lyase and cystathionine γ-synthase from *E. coli* (46).

Synthesis of homocysteine from cysteine. Cystathionine γ-synthase and cystathionine β-lyase respectively catalyze the two successive reactions of the transsulfuration pathway (Fig. 3):

$$\text{Cysteine} + \textit{O}\text{-acetylhomoserine} \rightarrow \text{cystathionine}$$

$$\text{Cystathionine} + H_2O \rightarrow \text{homocysteine} + \text{pyruvate} + \text{ammonia}$$

It is now well established that this pathway is fully active in yeast cells, since strains impaired in the sulfate assimilation pathway are able to grow in the presence of cysteine and wild-type strains of *S. cerevisiae* are capable of using cysteine as the sole sulfur source. The isolation of a mutant (*str2*) which is unable to use cysteine and which seems to have cystathionine β-lyase activity in vitro has been reported (41). However, no molecular data on this pathway are available. A homology search against the yeast genome revealed that it contains, in addition to the genes already assigned, five ORFs whose products show extensive sequence similarities to enzymes that catalyze sulfide incorporation and transsulfuration. These are the products of two ORFs, YHR112c and YJR130c, which show similarity to cystathionine γ-synthases, the products of two other ORFs, YFR055w and YGL184c, which show similarity to cystathionine β-lyases, and the product of YGR012w, which is similar to Cys4p.

Methionine Synthesis

Origin of the methyl group of methionine. De novo synthesis of methionine from homocysteine uses a methyl group which originates from single-carbon metabolism (107). In this metabolism, derivatives of tetrahydrofolate transfer one-carbon groups at the oxidation levels of methanol, formaldehyde, and formate to acceptor molecules. Single-carbon derivatives of tetrahydrofolate are required for the biosynthesis of methionine, purine nucleotides, and thymidylate, as well as for the synthesis of *N*-formylmethionine in the mitochondrion. *S. cerevisiae* possesses two complete sets of folate interconversion enzymes, one located in the cytosol and the other located in the mitochondrion (Fig. 7) (for a review, see reference 107).

Mutations impairing the steps of single-carbon metabolism, specifically devoted to methionine synthesis, are expected to result in methionine auxotrophic cells that, in addition, do not grow in the presence of either homocysteine or cysteine. Such mutations were previously identified as defining five complementation groups: *MET6*, *MET7*, *MET13*, *MET23*, and *MET24* (167). However, *MET23* and *MET24* have recently been shown to be equivalent to *MET7* and *MET6*, respectively (see Table 1) (36). Since *MET6* encodes homocysteine methyltransferase (see below), only *met7* and *met13* mutations are likely to affect single-carbon metabolism.

Only one step of the postulated single-carbon metabolism pathway is expected to be specific to methionine biosynthesis, i.e., the reduction of 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate (Fig. 7). This step is catalyzed by methylene tetrahydrofolate reductase (MTHFR). The *S. cerevisiae* genome contains two ORFs, *YGL125w* and *YPL023c*, whose products show extensive sequence similarity to mammalian MTHFR. *MET13* maps to the *YGL125w* region. Strains bearing a chromosomal deletion of the *YPL023c* ORF are prototrophic, whereas those with a chromosomal deletion of the *YGL125w* ORF require methionine for growth (130), just as *met13* mutants. The *YGL125w* ORF therefore, probably corresponds to *MET13*, which was mapped on chromosome VII (174) and should encode cytoplasmic MTHFR. The precise function of the YPL023c, however, is so far unknown.

met7 mutants were first reported as requiring both adenine and methionine for growth (107). However, strains bearing different alleles of *met7* have recently been studied, and no requirement for adenine could be observed (36). *MET7* has been cloned and shown to be identical to the *YOR241w* ORF from chromosome XV (36). Met7p has a predicted molecular weight of 62,000 and exhibits strong sequence similarity to tetrahydrofoylglutamate synthase. This enzyme catalyzes the extension of the glutamate chain of tetrahydropteroylglutamates (the tetrahydropteroylmonoglutamate [$H_4PteGlu_1$]):

$$H_4PteGlu_n + \text{glutamate} + \text{ATP} \rightleftharpoons H_4PteGlu_{n+1} + \text{ADP} + P_i$$

It has been observed that all enzymes of single-carbon metabolism can utilize in vitro the monoglutamate form of the tetrahydropteroyl coenzyme. However, in vivo studies with cultured Chinese hamster ovary cells showed that cells unable to synthesize the polyglutamate forms had nutritional requirements for the end products of one-carbon metabolism, i.e., thymine,

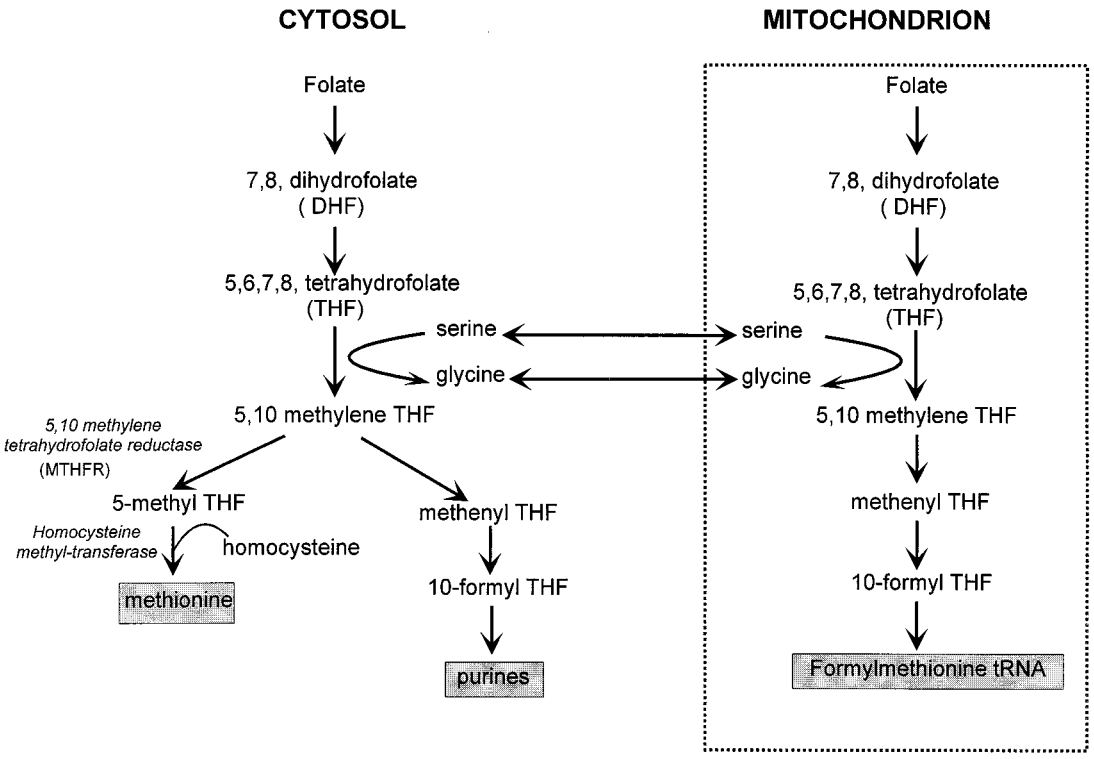

FIG. 7. Organization of the folate-mediated one-carbon metabolism (107).

methionine, and adenine (for a review, see reference 221). The fact that *met7* mutants appear to be only methionine auxotrophs suggests that in yeast, methionine synthase is the only enzyme that has an obligatory requirement for the polyglutamate form of the tetrahydropteroyl coenzyme.

Methionine synthesis. 5-Methyl-tetrahydrofolate homocysteine methyltransferase (homocysteine methyltransferase) synthetase catalyzes the reaction (Fig. 3).

$$\text{Homocysteine} + \text{5-methyltetrahydrofolate} \rightarrow \text{methionine} + \text{tetrahydrofolate}$$

E. coli synthesizes two distinct proteins with homocysteine methyltransferase activity. The MetH protein is a cobalamin (vitamin B_{12})-dependent enzyme that uses both monoglutamate and polyglutamate derivatives of methyl tetrahydrofolate as substrates. The MetE protein, which is cobalamin independent, has a strict requirement for the polyglutamate form of methyl tetrahydrofolate (for a review, see reference 191). In mammals, homocysteine methyltransferase activity is cobalamin dependent (35). In contrast, homocysteine methyltransferase from the plant *Catharanthus roseus* was expressed in *E. coli* and shown not to require cobalamin for activity (65). Since the pioneering biochemical work of Burton et al. (21), it is known that *S. cerevisiae* has only a cobalamin-independent homocysteine methyltransferase. Flavin pointed out that this is correlated with the fact that yeast is believed to be unable to synthesize adequate amounts of vitamin B_{12} and that the media used to grow *S. cerevisiae* do not contain vitamin B_{12} (71). Recently, it was formally shown that *S. cerevisiae* does not require vitamin B_{12} for growth (88), thereby confirming that the yeast homocysteine methyltransferase is cobalamin independent. This enzyme is encoded by *MET6* (56, 176). Met6p has a predicted molecular weight of 86,000 (Table 3) and is closely related to homocysteine methyltransferase encoded by the *metE* gene of *E. coli* (124). The growth of *met6* mutant cells on AdoMet (36) shows that methionine can be synthesized from AdoMet without involving homocysteine methylation. Since the reaction catalyzed by AdoMet synthetases is irreversible, this can be achieved only by a salvage pathway distinct from the methyl cycle (see below).

S-ADENOSYLMETHIONINE SYNTHESIS AND METABOLISM

In addition to methionine and cysteine syntheses, the sulfur pathway is responsible for the formation of AdoMet, a major constituant of intermediary metabolism. Since its discovery in 1951, AdoMet has been shown to be involved in such a great number of reactions that it is second only to ATP in cellular metabolism for the variety and number of reactions in which it serves as a cofactor (29).

AdoMet Synthesis

Methionine adenosyl transferase (AdoMet synthetase) catalyzes the formation of AdoMet at the expense of the complete dephosphorylation of one molecule of ATP in the presence of potassium and magnesium ions (Fig. 3):

$$\text{L-Methionine} + \text{ATP} \rightarrow \text{AdoMet} + \text{PP}_i + \text{P}_i$$

The mechanism of this reaction, in which a sulfonium compound is formed, is unusual in many respects (29). Maximum activity requires rather high concentrations of monovalent and divalent cations, and this reaction represents the only example of the utilization of the energy of the phosphate bond of ATP for the generation of an energy-rich sulfonium compound. In

addition, the reaction involves nucleophilic transfer of the 5′-deoxyadenosyl moiety of ATP to one of the free pairs of electrons of the thioether sulfur of L-methionine. It is the α and β phosphate groups of ATP that give rise to PP_i, whereas, the terminal phosphate is released as inorganic phosphate (P_i). Mudd (178) has provided convincing evidence that inorganic polyphosphate is an enzyme-bound intermediate that is cleaved by a tripolyphosphatase activity of AdoMet synthetase. AdoMet synthetase from yeast has been purified and studied in the past by several groups (48, 85, 178). However, only in 1977 was the existence of two AdoMet synthetases in *S. cerevisiae* recognized by Chiang and Cantoni (Table 3), who showed that purified AdoMet synthetase could be resolved into two active species by DEAE-cellulose chromatography (47). The presence of two different AdoMet synthetases in *S. cerevisiae* was confirmed by the identification of their encoding genes, *SAM1* and *SAM2* (44). In contrast to a *sam1 sam2* double-disruption mutation, which leads to AdoMet-requiring cells, single disruption of either *sam1* or *sam2* does not affect the growth, indicating that each enzyme is independently active (250).

The products of the *SAM1* and *SAM2* genes are 92% identical (250, 251) and closely resemble *E. coli* AdoMet synthase, encoded by *metK* (163). The structure of *E. coli* AdoMet synthase has been resolved and shown to be a homotetramer with two subunits forming a spherical tight dimer and pairs of dimers forming a peanut-shaped tetrameric enzyme (241, 242). A second AdoMet synthetase, which is encoded by *metX*, exists in *E. coli* (219, 220). The occurrence of multiple isomers of AdoMet synthetases in the same organism appears to be the rule. In plant cells, two AdoMet synthetase-encoding genes have been cloned from *Arabidopsis thaliana* (204, 205), while tomato contains at least four AdoMet synthetase-encoding genes (67). It is now known that two genes encode catalytically active subunits of AdoMet synthetase in mammals. The first, expressed in liver only, encodes a subunit found in two active AdoMet synthases, which are either a dimer or a tetramer of this subunit (25, 26). The second gene is expressed in many tissues and encodes a subunit that associates with a regulatory subunit in an oligomeric structure of unknown stoichiometry (96). The two catalytically active subunits of the human enzymes are less similar to one another than are the human and the rodent liver-specific enzymes (97, 98). No specific function has so far been assigned to each isoenzyme in *S. cerevisiae*. However, the *SAM2* gene is subject to specific transcriptional regulation, which results in increased synthesis of its encoded product at the end of the exponential growth phase (252).

AdoMet Utilization

Due to its electron-deficient trivalent sulfur, AdoMet is used in reactions involving extremely different chemical modifications. In fact, the three ligands attached to the sulfur atom are energetically equivalent and can be transferred to acceptor molecules, as postulated by Cantoni (30). The major consumption of AdoMet results from transmethylation reactions. Indeed, all types of organic molecules are susceptible to being methylated, and in the vast majority of these reactions, AdoMet is used as the methyl group donor. All transmethylation reactions involving AdoMet produce *S*-adenosylhomocysteine (AdoHC), in addition to the methylated acceptor. In other types of reactions, the carboxyaminopropyl group of AdoMet can be used for the synthesis of modified nucleotides in rRNA, or, after the decarboxylation of AdoMet, the aminopropyl group is used for polyamine synthesis. In these cases, methylthioadenosine (MTA) is formed as a by-product of the reaction. Both AdoHC and MTA can be recycled back to methionine (see below). AdoMet is also used as an amino group donor in the synthesis of pelargonic acid, a precursor of biotin (239). A new function of AdoMet was recently reported in the synthesis of queuine, a modified base of tRNAs of procaryotes and eucaryotes. In this reaction, AdoMet serves as ribosyl group donor (235). In plants, AdoMet is cleaved to MTA and aminocyclopropane carboxylic acid, a precursor of ethylene, the fruit-ripening hormone (243).

SULFUR SALVAGE PATHWAYS

S. cerevisiae contains several enzymatic systems that permit recycling of the by-products of AdoMet metabolism. These salvage pathways could allow high AdoMet utilization while maintaining the sulfur atom in its most reduced form (Fig. 8).

Methyl Cycle

The methyl cycle consists of reactions that recycle AdoHC, the sulfur compound formed during transmethylation reactions. Homocysteine, the direct precursor of methionine biosynthesis, is formed by hydrolysis of AdoHC. The earliest record of the methyl cycle is by Duerre, who demonstrated that when radioactive AdoHC (labeled in its homocysteine moiety) was added to the growth medium of wild-type *S. cerevisiae*, the radioactive label was found in intracellular homocysteine, cysteine, methionine, and AdoMet (64). Methyl cycle activity is further demonstrated by the ability of wild-type cells to use AdoMet as the sole sulfur source. Here, cells must synthesize both methionine and cysteine from AdoMet, and the reaction catalyzed by AdoMet synthetases is an irreversible reaction. In transmethylation reactions, AdoHC is produced in stoichiometric amounts and then degraded by AdoHC hydrolase, producing adenosine and homocysteine. The *S. cerevisiae* enzyme has been partially purified and characterized. The equilibrium of the AdoHC hydrolase reaction lies far toward the synthesis of AdoHC (119), suggesting that AdoHC hydrolysis is favored in vivo by the removal of homocysteine and adenosine by further metabolism. Homocysteine can then be metabolized to synthesize cysteine and methionine. It has also been shown that the presence of adenosine deaminase favors AdoHC hydrolysis in vitro (119). Yeast adenosine deaminase has been purified and characterized (156, 164), but its role in AdoHC metabolism has not been studied. However, the conversion of adenosine to AMP by adenosine kinase could be involved in this metabolism. Indeed, an adenine mutant of *S. cerevisiae* can grow on AdoMet and AdoHC, showing that adenosine can enter the purine nucleotide pool probably after its conversion to AMP by adenosine kinase (51, 118). A mutant impaired in adenosine kinase has been isolated (101). This mutant accumulates AdoHC, showing that adenosine kinase is likely to remove adenosine produced by AdoHC hydrolysis. No molecular analysis of AdoHC hydrolase, adenosine deaminase, or adenosine kinase has appeared. However, in the yeast genome is an ORF (*YER043c SAH1*) that may encode a polypeptide similar to human AdoHC hydrolase. An ORF potentially encoding a protein similar to adenosine deaminase of *E. coli* has been sequenced (161).

Methylthioadenosine Cycle

The MTA cycle recycles MTA into methionine (Fig. 8). This is achieved through an elegant biochemical process in which the ribose moiety of the adenosyl group of MTA gives rise to the four-carbon skeleton of methionine while conserving the methylthiol group. *S. cerevisiae* cells are normally unable to

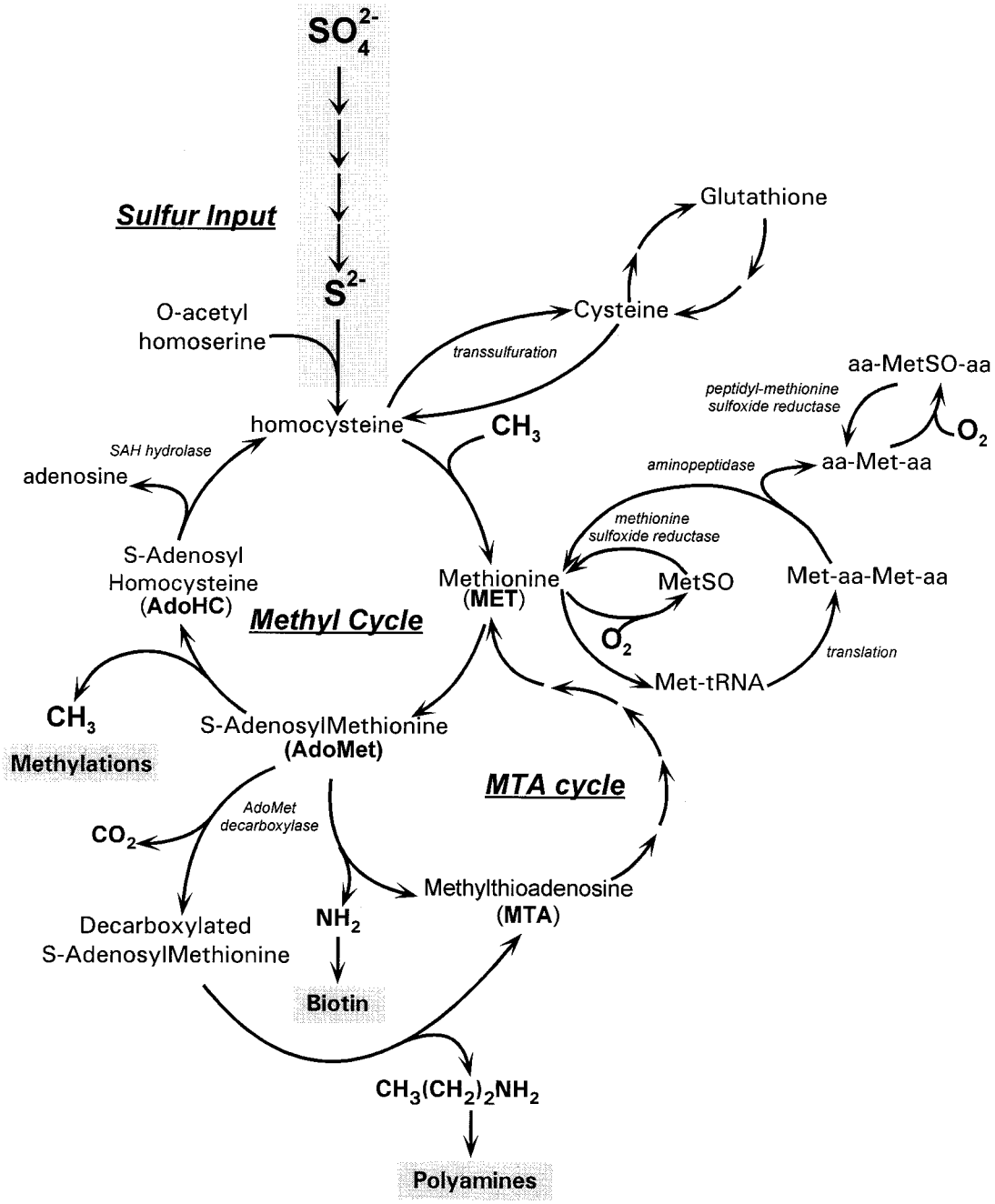

FIG. 8. Sulfur salvage pathways.

take up MTA from the growth medium. However, by using mutant cells able to transport this nucleotide, it has been shown that MTA can be used as a source of both purines and methionine (51). The presence of this cycle in *S. cerevisiae* is further supported by the ability of *met6* mutants to grow on AdoMet. These mutants are unable to synthesize methionine because they are impaired in homocysteine methyltransferase activity. However, in such mutants, cysteine is synthesized from AdoMet through AdoHC hydrolysis and the transsulfuration pathway whereas methionine is synthesized from AdoMet through the MTA salvage pathway. This pathway has been well characterized in *Klebsiella pneumoniae*, beginning with nucleosidase cleavage of MTA to adenine and 5-methylthioribose (63). The C-1 hydroxyl group of the ribose moiety of methylthioribose is in turn phosphorylated by methylthioribose kinase, producing methylthioribose 1-phosphate (69). After isomerization and dehydration to the diketo intermediate, the molecule is oxidatively decarboxylated to 2-keto-4-methylthiobutyrate, the immediate precursor of methionine (5).

Purification of the two first enzymes (nucleosidase and kinase) from *K. pneumoniae* has been reported (53). It is noteworthy that *E. coli* is incapable of methionine salvage from methylthioadenosine because it lacks methylthioribose kinase (cited in reference 53). In plants, the MTA cycle is involved in recycling MTA generated during ethylene biosynthesis and is called the Yang cycle (243).

TABLE 4. Sulfur compound uptake: structural genes

Gene	Alternate name	Chromosome	Protein	Mutant phenotype[a]
SUL1	*YBR294w*	II	Sulfate transporter	Selenate resistant
SUL2	*YLR092w*	XII	Sulfate transporter	Selenate resistant, *sul1 sul2* double mutant is methionine auxotroph
MUP1	*YGR055w*	VII	Methionine permease (high affinity)	Ethionine sulfoxide resistant
MUP2		?	Methionine permease (low specificity)	No phenotype
MUP3	*YHL036w*	VIII	Methionine permease (low affinity)	No phenotype
SAMP3		?	AdoMet permease	*S*-Adenosylethionine resistant, cannot use AdoMet as a sulfur source

[a] See references 37, 100, and 238.

Amino-Terminal Methionine Recovery

Apart from its function as AdoMet precursor, methionine is a protein constituant. In all living cells, protein synthesis is initiated with either methionine (in the cytosol of eukaryotes) or formylmethionine (in prokaryotes, mitochondria, and chloroplasts). When formylmethionine is used, the formyl group is subsequently removed by a deformylase, leaving methionine. Therefore, in both cases, nascent polypeptides begin with a methionine residue, which may be removed by aminopeptidases. This reaction allows the recovery of methionine and therefore can be considered a sulfur salvage pathway. The amino-terminal methionine is removed in *S. cerevisiae* if the penultimate residue is small and uncharged (173), and two methionine aminopeptidases have been characterized. One is encoded by *MAP1* and contains an N-terminal zinc finger. Disruption of *MAP1* does not cause cell death, although it dramatically slows growth. The second aminopeptidase is encoded by *MAP2*. Map1p and Map2p are 22% identical. *map2* null cells are viable but display slow growth. The *map1 map2* double mutant is inviable, showing that removal of the initiator methionine is an essential function (151).

UPTAKE OF SULFUR COMPOUNDS

Sulfate

Sulfate is among the most abundant anion macronutrients in cells after phosphate and is the major sulfur source in many organisms. Like all inorganic nutrients, sulfate is transported into cells by highly specific membrane transport systems. It is after accumulation that sulfate is enzymatically reduced to sulfide by the sulfate assimilation pathway and then incorporated into organic compounds (see above). Sulfate transport has been studied in whole plants (50, 141, 143), excised organs (49, 55), and isolated cells (212, 237). More recently, kinetic studies of sulfate transport into plant right-side-out purified plasma membrane vesicles have shown that it occurs by sulfate/proton cotransport (91), as it does in *S. cerevisiae* (215). This differs from sulfate transport in animal plasma membrane vesicles, where it is an anion-exchange process (170) or a sodium/sulfate cotransporter (23).

From preliminary genetic work, it was proposed that sulfate transport in yeast occurs via two independent systems (20). An *S. cerevisiae* mutant impaired in sulfate transport has been isolated, and the corresponding *SUL1* gene has been cloned (236). Systematic analysis of mutants resistant to toxic analogs of sulfate, selenate, and chromate identified three unlinked genes whose products are specifically involved in sulfate uptake: *SUL1*, *SUL2*, and *SUL3* (Table 4). Sul1p and Sul2p are 62% identical. Both are predicted to be integral membrane proteins possessing 11 and 10 transmembrane domains, respectively. These proteins are similar to the mycelial sulfate transporter (encoded by *cys-14*) of *N. crassa* (112). Sulfate transport kinetic studies performed with *SUL1* or *SUL2* deletion mutants showed that both genes encode high-affinity sulfate transport proteins (Table 5). The *SUL3* product participates in the transcriptional regulation of *SUL2* by an unknown mechanism (37). It is not known if the *SUL1* and *SUL2* genes are the same as the *CHR* and *SEL* genes characterized previously (20).

The uptake of sulfite by microorganisms has received scant attention. In aqueous solutions, sulfite exists as a mixture of three forms, whose proportions depend on pH. At low pH (<1.77), SO_2 is predominant; at high pH (>7.2), it exists largely as sulfite ion, SO_3^{2-}; and at intermediate pH, these two forms coexist in various proportions with bisulfite ion HSO_3^-. The first report on sulfite transport concluded that only SO_2 could enter the cells via an active carrier-mediated process (160). A more recent study reached the same conclusion for cells grown at pHs ranging from 3 to 5. However, it was suggested in this study that SO_2 is transported by simple diffusion (240).

Methionine

In yeast, each amino acid is transported by specific and nonspecific permeases. General amino acid permease (Gap1p) transports all naturally occurring L-amino acids found in proteins, as well as related compounds such as ornithine and citrulline, several D-amino acids, and toxic amino acid analogs (261). However, Gap1p functions only under particular growth conditions. For instance, in the presence of an efficient nitrogen source, such as ammonium, Gap1p activity is absent. With ammonium ions as the nitrogen source, amino acids are transported by specific permeases as confirmed by both biochemical and genetic data. Several genes encoding specific high-affinity permeases have been cloned and sequenced. They all encode integral membrane proteins with 12 putative membrane-spanning regions and are quite similar, suggesting that they are

TABLE 5. Uptake of sulfur compounds: kinetic parameters[a]

Transporter	Substrate	J_{max} (nmol/min/mg [dry wt])	K_T (mM)
Sul1p	Sulfate	ND[b]	0.0045
Sul2p	Sulfate	ND	0.010
Mup1p	Methionine	17	0.013
Mup2p	Methionine	ND	0.26
Mup3p	Methionine	52	1.0
?	Cysteine	1.25[c]	0.083
Samp3p	AdoMet	3	0.0033

[a] See references 37, 100, 183, and 196.
[b] ND, not determined.
[c] Expressed in nanomoles per minute per unit of optical density at 600 nm.

TABLE 6. Additional methionine auxotrophic mutants

Gene	Alternate name(s)	Chromosome	Protein[a]	Mutant phenotype[a]
MET18	*MMS19*, *YIL128w*	IX	Leucine-rich motif repeats	Methionine auxotroph, MMS sensitive[b]
MET19	*ZWF1*, *YNL241c*	XIV	G6PDH[b]	Methionine auxotroph, sensitive to oxidizing agents
MET27	*VPS33*, *SLP1*, *VPT33*, *VPL25*, *VAM5*, *CLS14*, *PEP14*, *YLR396c*	XII	Vacuolar sorting protein	Methionine auxotroph, salt sensitive
SOD1	*YJR104c*	X	Cu,Zn superoxide dismutase	Methionine auxotroph, lysine auxotroph, superoxide sensitive
TRX1	*YLR043c*	XII	Thioredoxin	
TRX2	*YGR209c*	VII	Thioredoxin	*trx1 trx2* double mutant is methionine auxotroph

[a] See references 34, 102, 115, 142, 179, and 247.
[b] MMS, methanesulfonic acid methyl ester; G6PDH, glucose-6-phosphate dehydrogenase.

members of a single gene family (for a review, see reference 2). The existence of specific high-affinity and low-affinity L-methionine permeases has been reported (80). However, a recent analysis shows that methionine is transported by one high-affinity and two low-affinity permeases (100). Mutants resistant to ethionine sulfoxide, a toxic analog of methionine, are impaired in the high-affinity methionine permease (*MUP1*) (Table 4). *MUP1* encodes an integral membrane protein with 13 putative membrane-spanning domains. ORF *YHL036* from chromosome VIII encodes a polypeptide similar to Mup1p and was demonstrated to encode the very low-affinity methionine permease (Table 5); it was called Mup3p. Mup1p and Mup3p are only distantly related to other known amino acid permeases and thus appear to define a new family of amino acid transporters. The second low-affinity methionine permease (encoded by *MUP2*) is less specific than the others. The *MUP2* gene has not yet been cloned and characterized (100).

Cysteine and AdoMet

Cysteine transport in *S. cerevisiae* has been characterized. The kinetic study reveals only one cysteine permease, which is inhibited only by homocysteine and methionine (196) (Table 5).

Unlike enteric bacteria, *S. cerevisiae* is capable of actively transporting AdoMet. AdoMet uptake has been characterized, and its kinetic parameters have been determined (Table 5). Surprisingly, AdoMet uptake appears to be strongly inhibited by the unrelated amino acid leucine. Mutants defective in the *SAM-P3* gene have been isolated and are impaired in AdoMet uptake, but no molecular data on this transport system are available (183, 238).

OTHER METHIONINE-REQUIRING MUTANTS

Vacuolar Mutants

A potent genetic screen, based on a gene fusion expressing the *xylE* gene of *Pseudomonas putida* under the control of the promoter region of *MET25*, was used to isolate mutants impaired in transcriptional regulation of the *MET* genes. During this study, a new mutation (*met27*) was isolated. *met27* mutant cells require methionine for growth and display a modified transcriptional regulation of sulfur amino acid metabolism (102). The *MET27* gene was cloned and shown to be identical to *VPS33* (Table 6), whose product is implicated in the biogenesis and inheritance of vacuoles (9, 257). *VPS33* encodes a protein of 691 amino acids that does not appear to be an integral component of the vacuole. Vps33p has two regions of similarity to ATPases that could be involved in nucleotide binding. It has been suggested that Vps33p acts in the transport of proteins from the Golgi to the vacuole (9).

At least five classes of vacuolar morphologies exist among the *vps* mutants (for reviews, see references 115 and 211). *vps33* (*met27*) mutants (class C) exhibit the most extreme vacuolar abnormalities; they possess no apparent vacuole but accumulate small vesicles (9, 257). Although *vps33* (*met27*) mutations result in pleiotropic phenotypes, their methionine auxotrophy was not initially observed (106, 114, 190, 257, 258). Other class C vacuolar mutants do, however, require methionine for growth (102).

AdoMet has been shown to be distributed in exchangeable cytosolic and vacuolar pools (68), with the latter accounting for up to 70% of the total intracellular AdoMet (227). The fragmented vacuole-like vesicles seen by electron microscopy in *met27* mutants have been interpreted as an indication of lost AdoMet storage capacity. Because AdoMet is the effector in the negative regulation of sulfur amino acid metabolism (see below), an increase of the cytosolic AdoMet concentration would modify *MET* gene expression such that addition of methionine to the growth medium would be required to overcome the imbalance of sulfate flux (102).

Glucose-6-Phosphate Dehydrogenase Mutants

met19 mutants were isolated as methionine auxotrophs (167) that could grow on methionine, cysteine, homocysteine, and AdoMet but not sulfide as the sulfur source. Cloning and sequencing of the *MET19* gene revealed that it encodes glucose-6-phosphate dehydrogenase, the first enzyme of the pentose phosphate pathway (Table 6) (247). Glucose-6-phosphate dehydrogenase mutants (*zwf1*) have been isolated, but the methionine auxotrophy was not noticed (154). The reasons for this methionine auxotrophy are still obscure (see the next section and reference 99). However, the physiological link existing between glucose-6-phosphate dehydrogenase and sulfur amino acid metabolism is certainly important, because *MET19* (*ZWF1*) transcription is repressed by increased intracellular AdoMet concentrations (247). That regulation is dependent on the negatively acting Met30p (see below and reference 249). In addition, mutations within *MET19* (*ZWF1*) result in sensitivity to oxidative stress (129).

Superoxide Dismutase Mutants

Mutants with mutations in *SOD1* (Table 6) (Cu,Zn superoxide dismutase) exhibit a requirement for methionine and lysine. Methionine can be replaced by cysteine, sulfide, or thiosulfate but not sulfite when *sod1* cells are aerobically grown in synthetic medium (34). It was shown that *sod1* mutants have

TABLE 7. Regulatory genes

Gene	Alternate name(s)	Chromosome	Protein	Mutant phenotype[a]
MET4	*YNL103w*	XIV	bZIP	Methionine auxotroph
MET28	*YIR017c*	IX	bZIP	Organic sulfur auxotroph
CBF1	*CEP1*, *CPF1*, *YJR060w*	X	bHLH	Methionine auxotroph, benomyl sensitive, thiabendazole sensitive
MET30	*YIL046w*	IX	WD40 repeats, F-box	Selenate sensitive under repressive growth conditions
MET31	*YPL039w*	XVI	Zinc finger protein	
MET32	*YDR253c*	IV	Zinc finger protein	*met31 met32* double mutant is methionine auxotroph
SUL3		?	?	Resistant to selenate

[a] See references 17, 28, 37, 135, 248, and 249.

all of the enzymes necessary for sulfate reduction and assimilation and that sulfite does not support growth because it is extremely toxic to *sod1* mutants (34). The sulfite toxicity in *sod1* mutants can be explained by the reduction of sulfite by superoxide, $O_2^{\cdot-}$, producing the sulfur trioxy radical, $SO_3^{\cdot-}$, which is as reactive as OH$^{\cdot}$. In this model, reversal of the sulfite toxicity by methionine would result from the repression of sulfate assimilation by AdoMet synthesized from methionine. Thus, a *sod1* mutant would require methionine for growth not because of an impairment in the biosynthetic pathway but because methionine turns off the assimilation of sulfate (for a review, see reference 24). This hypothesis is questionable, however, because *sod1* mutants can grow on sulfide, thiosulfate, and cysteine, although none of these sulfur compounds repress sulfate assimilation (34).

Recently, a metabolic connection between Sod1p and the pentose phosphate pathway has been suggested: both are critical for maintenance of the cellular redox state (99). A careful phenotypic study of *ZWF1* and *SOD1* null mutants led these authors to propose that both possess the same block in sulfate assimilation: lower NADPH availability for NADPH-dependent reduction of PAPS and sulfite.

Mutants with Mutations in the *MMS19* Gene

met18 mutants, isolated (Table 6) as methionine auxotrophs, are impaired in sulfite reductase activity (167). Although no sulfite reductase activity was found in vitro, some was detected in vivo, showing that *met18* mutants possessed the enzymes necessary for reduction and sulfate assimilation (244). The *mms19* mutants were isolated as cells sensitive to the alkylating agent methyl methanesulfonate (208). *mms19* mutants require methionine for growth (142), and the *MMS19* and *MET18* genes were shown to be identical. Mms19p is a multifunctional protein, involved in both nucleotide excision repair and RNA polymerase II transcription (142). *MMS19* (*MET18*) encodes a protein of 1,032 residues which shows no strong homology to any other protein in the databases aside from 15 repeats of a leucine-rich motif, which is found in various eucaryotic regulatory proteins (142).

TRANSCRIPTIONAL REGULATION OF THE SULFUR AMINO ACID PATHWAY

The following part of this review will focus on the specific regulation that leads to the repression of *MET* gene expression when high concentrations of methionine are added to the growth medium. The genes encoding factors involved in the control of expression of the *MET* genes are listed in Table 7.

Sulfur Amino Acid Metabolism and General Control of Amino Acid Biosynthesis

A cross-pathway regulation of amino acid biosynthesis exists in *S. cerevisiae*. Starvation for any one of several amino acids increases the expression of genes encoding enzymes of multiple amino acid biosynthetic pathways. Studies of this regulatory response (general control of amino acid biosynthesis) clearly show it to be dependent upon the short ATGA(C/G)T CAT sequence which is repeated upstream of every regulated gene and which is the DNA binding site of the transcriptional activator Gcn4p (94). The biosynthesis of sulfur amino acids is largely immune to general control; in most cases, *MET* gene promoters do not contain the ATGA(C/G)TCAT sequence. However, this sequence does occur upstream of *MET4*, *MET5*, *MET6*, and *MET16*. In these cases, expression responds to amino acid starvation in a Gcn4p-dependent manner (175, 176, 189). Gcn4p-mediated derepression occurs in response to the accumulation of uncharged tRNAs (94). Cherest et al. reported that the synthesis of some methionine biosynthetic genes was not repressed by the addition of methionine to the growth medium in a strain bearing a *mes1* mutation which impairs methionine tRNA synthetase (45). This could be the result of either an accumulation of uncharged $tRNA^{Met}$ triggering the general control or an impairment of the specific control by AdoMet (see below). The second hypothesis is more likely, since the AdoMet pool reached in this strain by growth in the presence of methionine is low compared to that in the wild type and addition of AdoMet to the growth medium of the *mes1* mutant restores the repression of enzyme synthesis (45).

AdoMet Response

Biosynthesis of sulfur amino acid in yeast has long been known to be specifically and negatively regulated. Synthesis of the enzymes required for sulfate assimilation, as well as methionine and cysteine synthesis, is repressed by the addition of methionine or AdoMet to the growth medium (38). Typically, wild-type cells grown in the presence of 1 mM L-methionine express less than 10% of the enzymatic activities measured after growth in ≤0.05 mM methionine. In subsequent sections, 1 mM L-methionine will be designated repressive while ≤0.05 mM methionine will be considered nonrepressive. In contrast, addition of high concentrations of either homocysteine or cysteine does not significantly affect the synthesis of these enzymes. Several early observations favored the hypothesis that negative regulation, observed when cells were grown in the presence of 1 mM L-methionine, was a consequence of its rapid conversion to AdoMet, thereby increasing the intracellular pool of the latter (42, 43). Definitive support for this hypothesis was shown by using a strain whose *SAM1* and *SAM2* genes

were both disrupted. In the absence of AdoMet synthase activities, these cells are unable to convert excess external methionine into AdoMet. Since the growth of these cells in the presence of 1 mM L-methionine does not lead to repression of enzyme biosynthesis, it was concluded that an increased intracellular AdoMet concentration would be the most proximal signal for repression (250).

Molecular cloning and analysis of structural genes subjected to AdoMet-mediated negative regulation demonstrated that their expression is transcriptionally regulated (40, 218). The sulfur amino acid metabolic pathway may therefore be considered an example of a small gene network, whose transcription is turned off in response to increased concentrations of the pathway end product. However, it must be noted that transcriptional repression is less significant for *MET6*, *SAM1*, and *SAM2* (176, 252). Even in the presence of high methionine, homocysteine methyltransferase and AdoMet synthase activities are required for the necessary synthesis of AdoMet and recycling of the reduced sulfur atom. Moreover, transcription of *SAM2* overrides the AdoMet-mediated repression, resulting in increased Sam2p at the end of the exponential growth phase (252).

Identification of the *cis*-Acting Regulatory Elements at Structural Genes

The first identification of DNA sequences that might mediate specific regulation of the *MET* genes was established through deletion analysis of the *MET25* upstream region after growth under nonrepressive and repressive conditions (246). *MET25* was chosen for this analysis because both the 5′ end and half-life of its mRNA were known. The *MET25* transcript starts 40 nucleotides upstream from the ATG codon, and its half-life is about 10 min (218). It must further be noted that to take into account the possible influence of the chromatin structure on *MET25* gene expression, the effects of the small internal deletions were analyzed not on plasmid constructs but in a chromosomal locus. Analysis of the *MET25* upstream region identified two important regions. The first, centered around CACGTG palindromes, was required for derepression of *MET* gene expression when intracellular AdoMet concentrations are low. As shown below, this *cis*-acting element is the binding site for the heteromeric transcription activation complex, Cbf1p-Met4p-Met28p (134, 135). The second functional sequence is the consensus sequence AAANTGTG. This element was first characterized as being required for full repression of *MET25* at high intracellular AdoMet concentrations. This DNA sequence was subsequently shown to bind two specific factors, Met31p and Met32p, whose functions are not yet fully understood (17).

Identification of the TCACGTG Sequence as a Positive Regulatory DNA Element

Deletions of *MET25* between nucleotides −320 and −270 (numbered relative to the ATG codon) lead to greatly diminished transcription. These drastic effects, resulting in less than 10% of the homocysteine synthase activity observed in wild-type cells, were observed under both nonrepressive and repressive growth conditions. Additional deletions localized the functional sequences to two copies of the sequence CACGTG. Their participation in transcription activation was further supported by cloning an oligonucleotide containing one of these copies together with its adjacent nucleotides (spanning nucleotides −310 to −288 of the *MET25* upstream region) in place of several of the deletions described above. In each case, CACGTG and its adjacent nucleotides allowed *MET25* transcription to be reacquired (246). The functional relevance of this sequence in the transcriptional regulation of sulfur metabolism was further supported by the finding of one or two copies of closely related sequences in the upstream regions of all the pathway structural genes (Fig. 9) except *SAM1*. In most cases, the 5′ adjacent nucleotide was T, suggesting a consensus sequence, TCACGTG.

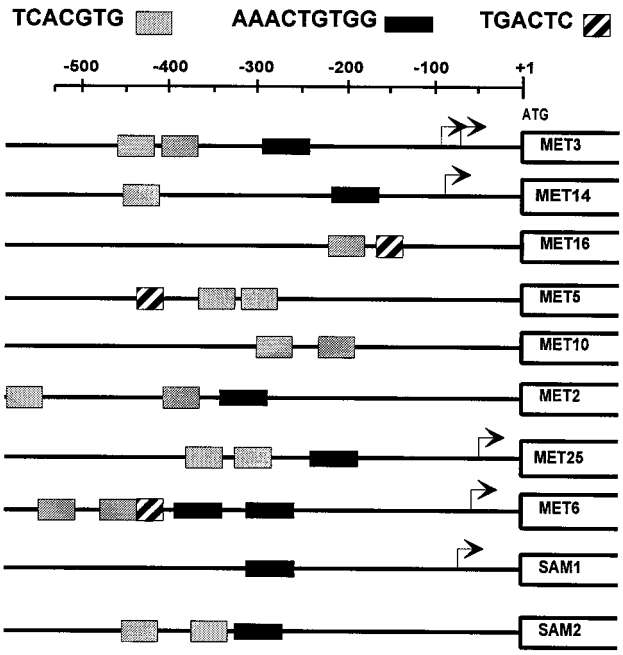

FIG. 9. Organization of the upstream regions of the *MET* and *SAM* genes. The data are compiled from references 125, 245, and 246 and sequences in the *Saccharomyces* Genomic Data (Stanford University).

The putative function of the TCACGTG sequence upstream of *MET25* was further substantiated by construction of *MET25-CYC1* hybrid promoters. *MET25* sequences containing the TCACGTG element and its adjacent nucleotides were inserted into a *CYC1* heterologous expression vector (248). These experiments showed that the TCACGTG sequence alone was not capable of activating the expression of the reporter gene. In contrast, an 18-bp element, containing the *MET25* TCACGTG sequence and its nine 5′ adjacent nucleotides (spanning nucleotides −310 to −294 of the *MET25* upstream region), supports reporter gene transcription. Such a construct was also shown to respond to increased concentrations of intracellular AdoMet: the β-galactosidase activity measured in cells grown under repressive conditions was sixfold lower than that in cells grown under nonrepressive conditions (248).

All these experiments suggest that TCACGTG is necessary but not sufficient for transcriptional activation, which requires the nucleotides present near the TCACGTG element. This conclusion was supported by mutational analysis of the *MET16* promoter, which identified a mutation (*met16-33*) that modified two nucleotides adjacent to the TCACGTG and resulted in the impairment of transcription activation (189). Surprisingly, the DNA element TCACGTG was also known to participate in chromosome segregation: TCACGTG is the CDE1 element (for centromere determining element 1) in all yeast centromeric regions (57, 92, 93).

Cbf1p Is Involved in Chromosome Segregation and Transcriptional Activation of the *MET* Genes

Following elucidation of the yeast centromere structure, proteins binding to the CDE1 element in vitro were isolated (7, 19, 27, 104). Partial amino acid sequences of these purified species allowed cloning of their cognate genes. These studies demonstrated that the apparently distinct protein species were all encoded by the same gene, *CBF1* or *CEP1* or *CPF1* (8, 28, 171), suggesting that the different isolated species were degradation products. Cbf1p consists of 351 amino acids with a calculated molecular weight of 40,000. Cbf1p contains a basic helix-loop-helix (bHLH) domain, a motif common to many eucaryotic DNA binding factors (28). As expected, the bHLH domain of Cbf1p was proven to be required for binding to the TCACGTG sequence (171, 172). Several members of the bHLH protein family have been shown to contain an additional leucine heptad repeat (leucine zipper domain) following the second helix of the bHLH domain and were therefore designated bHLH-LZ factors (16). In most cases, the leucine zipper was shown to participate in the dimerization of such factors (16). Structural analysis of the carboxy-terminal domain of Cbf1p predicted some resemblance to a leucine repeat (62), showing that it shared functional characteristics with the bHLH-LZ DNA binding factors. Contradictory results, however, were obtained through mutagenesis of Cbf1p: Masison et al. concluded from the mutations they obtained that the bHLH domain of Cbf1p is sufficient for dimerization (166). In contrast, Dowell et al. reported that the heptad repeat found at the end of the bHLH domain is responsible for Cbf1p dimerization, although these authors did not exclude the possibility that the bHLH domain itself was required for optimizing this function (62). Upon inactivation of the chromosomal copy of *CBF1*, no CDE1 binding activity can be detected in cell extracts, suggesting that Cbf1p represents the major TCACGTG binding activity in *S. cerevisiae* (171).

The analysis of *cbf1* mutants supported the proposed implication of Cbf1p in chromosome segregation and sulfur amino acid metabolism. These mutants exhibit an 8- to 10-fold increase of mitotic and meiotic chromosome loss as well as a nutritional requirement for organic sulfur (8, 28, 171). In addition, cells lacking Cbf1p are hypersensitive to drugs that disrupt the microtubule function (thiabendazole and benomyl) (28). It is not clear, however, whether this phenotype is specific or the consequence of impairing chromosome segregation.

cbf1 disruption strongly decreases *MET16* transcription when cells are grown under nonrepressive conditions (248). Moreover, these cells lack sulfate permease activity (248). Taken together with identification of TCACGTG as a *cis* element required for *MET25* gene expression, these results seemed to confirm that transcriptional activation of the *MET* genes should require the binding of Cbf1p to their upstream regions. The accumulated evidence suggests that direct, specific Cbf1p binding upstream of the *MET* genes is required for its function during transcriptional activation. This model was elegantly supported by domain swap experiments (58). Dang et al. (58) engineered hybrid Cbf1 proteins in which the basic region of Cbf1p was replaced by that of either USF or AP4. USF and AP4 are two mammalian bHLH DNA binding factors that recognize the palindrome CACGTG and CAGCTG, respectively. The Cbf1-USF hybrid protein recognizes the CACGTG sequence, and its expression in *cbf1*-disrupted cells restores methionine prototrophy. In contrast, the Cbf1-AP4 hybrid protein is unable to bind to the CACGTG sequence and does not restore methionine prototrophy when expressed in cells lacking Cbf1p. Furthermore, when methionine prototrophic revertants were selected from cells expressing the Cbf1p-AP4 hybrid protein, they all contained an amino acid change in the basic AP4 portion of the hybrid (58). Despite these observations, Mellor and coworkers proposed that Cbf1p might function in a non-DNA bound form during transcriptional activation of the *MET* genes while a DNA bound form acted at centromeres (109, 172). This conclusion arose mainly from the analysis of one Cbf1p derivative (a Glu231Ala replacement), which failed to bind in vitro to the CACGTG sequence but did not cause methionine auxotrophy when expressed in yeast (172). This result was challenged by the work of Foreman and Davis, who analyzed three mutations within the basic region of Cbf1p that all resulted in the replacement of the Glu231 residue (Glu231Gly once and Glu231Val twice). In each case, the resulting Cbf1p derivative did not bind the CACGTG sequence and did not permit growth in the absence of methionine when expressed in Cbf1p-deficient cells (73). Moreover, the results of a systematic mutational analysis of Cbf1p fully support the view that a DNA-bound form of Cbf1p is required for transcriptional activation of the *MET* genes (166).

In spite of the numerous studies of Cbf1p function, its role in the transcriptional activation of the *MET* genes was not understood. Cbf1p did not appear to behave as a typical transcriptional activator. When expressed in yeast, LexA-Cbf1 fusion proteins were unable to activate transcription of a reporter gene placed downstream of LexA operators. The same proteins were shown to be fully able to complement methionine auxotrophy resulting from inactivation of the chromosomal *cbf1* gene, prompting the proposition that Cbf1p might positively influence the *MET* gene transcription by modulating the chromatin structure in their upstream region (248). However, cells that lack Cbf1p exhibited changes in the chromatin structure upstream of *MET16*, which were limited to the immediate nucleotides adjacent to TCACGTG (189). A nearly identical result was obtained for the *MET25* promoter (109). Furthermore, important modifications of the overall chromatin structure were not observed upstream of *MET16* and *MET25* upon transcriptional activation (109). Thus, Cbf1p does not seem to function in modifying the phasing of the nucleosomes. In fact, the molecular mechanism by which Cbf1p functions at *MET* promoters was only recently deciphered when a transcriptional activation complex associating Cbf1p with Met4p and Met28p was demonstrated (135): Cbf1 appears to function by tethering the specific transcription activator Met4p to the promoter (see below). However, it must be emphasized that inactivation of the *CBF1* gene does not impair the transcription of all genes of the sulfate assimilation pathway to the same extent (137, 248). While *MET10*, *MET14*, and *MET16* gene expression was shown to depend strictly on the presence of active Cbf1p, Northern blot experiments demonstrated that *MET3* and *MET25* transcription reached half of the wild-type levels in a *cbf1* mutant (137).

MET4 Encodes the Main Transcription Activator of the Sulfate-Assimilating Pathway

Although the above studies did not permit an understanding of how Cbf1p functions in transcriptional activation of sulfur amino acid metabolism, they strengthened the hypothesis that transcription activation could not be achieved through Cbf1p alone. Therefore, the molecular mechanisms sustaining this regulation should involve other *trans*-acting factors. Systematic physiological analysis of methionine auxotrophs identified mutations at the *MET4* locus that rendered cells selenate resistant and unable to grow on inorganic sulfur sources or on cysteine (244). Enzyme assays on *met4* cell extracts revealed undetect-

able levels of all enzymes required for sulfate assimilation (248). *met4* mutations also impaired sulfate transport. As expected from the enzyme assays, Northern experiments demonstrated that transcriptional activation of *MET2*, *MET3*, *MET5*, *MET14*, *MET16*, *MET10*, and *MET25* genes does not occur in *met4* mutants (138, 175, 245, 248). *MET4* encodes a rather large protein of 666 amino acid residues (248). It was suggested that *MET4* translation might not start at the first in-phase ATG codon (175). However, as noted by these authors, the subclones lacking the first ATG codon do not complement a *met4* chromosomal deletion as well as the entire clone does. Moreover, subsequent construction of *MET4-lacZ* gene fusions have shown that the first in-phase ATG codon is efficiently transcribed (138). Sequence analysis revealed that Met4p contains a carboxy-terminal repeat of five leucine heptads preceded by a region rich in basic residues (248). Such a motif, called the basic leucine zipper motif (bZIP), has been found in many regulatory proteins from higher eucaryotes and fungi (139). Numerous biochemical and molecular studies have shown that bZIP domains constitute bipartite motifs participating in specific DNA binding through the basic region and dimerization through the leucine zipper region (1). The X-ray structure of the Gcn4p leucine zipper confirms that dimerization results from side-by-side packing of the leucine residues as well as of polar residues found at specific positions within the heptads (66). The Met4p region encompassing the basic region and leucine zipper is free of proline and glycine residues and is predicted to be fully folded into an α-helical secondary structure (248). However, Met4p differs from the vast majority of the other bZIP factors by the unusual sequence of its basic region. Sequence alignments made with known bZIP factors suggest that their basic regions may be divided into two subdomains rich in basic amino acids separated by a linker containing invariant asparagine and alanine residues. In contrast, the basic domain of Met4p is continuous and lacks these conserved asparagine and alanine residues. However, expression of truncated Met4p derivatives suggests that the bZIP domain was responsible for targeting of Met4p to the DNA. In particular, removal of the bZIP domain of a LexA-Met4p fusion protein eliminates its ability to complement the methionine requirement of a *met4* deletion mutant (248).

LexA-Met4p fusion proteins were used to demonstrate that Met4p is a strong transcriptional activator (248) and that this function is inhibited by increases in the intracellular AdoMet concentration (see below). Moreover, assays of *MET25-CYC1-lacZ* reporter constructs in *met4*-disrupted cells had shown that TCACGTG-bound Met4p was required for reporter gene expression (248).

Met28p, a Second bZIP Factor, Is Required for Full Induction of the *MET* Genes

The *MET28* gene was isolated as a mutation conferring both organic sulfur auxotrophy and selenate resistance (37). Initial enzyme assays revealed the pleiotropic effects of the *MET28* mutations which decreased the enzyme activities required for sulfate assimilation by 2- to 10-fold. *MET28* encodes a small protein of 166 amino acid residues which contains half a bZIP motif in its carboxy terminal (135). Unlike that of Met4p, the bZIP domain of Met28p can be aligned with other known bZIP regions. In fact, Met28p is closely related to the C/EBP subclass of bZIP factors having a 7-amino-acid segment between the leucine zipper and basic region. The leucine zipper of Met28p exhibits, like that of Met4, the classic 4-3 repeats of hydrophobic residues. Northern blot experiments revealed that a *met28* chromosomal deletion lowers maximal *MET3*, *MET10*, *MET14*, and *MET16* transcription compared to the wild type. By contrast, *MET25* gene transcription remained unaffected by the *met28* mutation (135). Met28p therefore appears to function as a positive effector of transcription of several *MET* genes when the intracellular AdoMet concentration is low. The role of Met28p was subsequently studied with a LexA-Met28p fusion protein. In wild-type cells, a LexA-Met28p fusion protein stimulates, albeit at a low level, the expression of a *lacZ* reporter gene containing upstream LexA-binding sites. This reporter gene expression is lost in a *met4*-disrupted strain. These results suggest that Met28p is devoid of an intrinsic transcription function. Presumably, in wild-type cells, the LexA-Met28p fusion protein activates the reporter gene by recruiting Met4p to DNA regions containing the LexA operators (135).

Identification of the Cbf1p-Met4p-Met28p Complex

The above results suggest that at least three positively acting factors are required for maximal *MET* gene transcription whereas only one type of positive *cis*-acting sequence was identified in their upstream regions. This is particularly important for *MET16* since its promoter contains only one TCACGTG sequence. To address this issue, gel mobility shift assays were performed with a radiolabeled *MET16* probe and extracts were prepared from wild-type cells or mutant cells lacking Cbf1p, Met4p, or Met28p (135). Two protein-DNA complexes were obtained with wild-type extracts; one corresponded to the Cbf1p binding alone. The second, more slowly appearing one was present in wild-type extracts but not in extracts from cells bearing a chromosomal deletion of *CBF1*, *MET4*, or *MET28*. To assess whether the high-molecular-weight complex contained each of the three factors, extracts were then prepared from cells lacking Cbf1p, Met4p, or Met28p but containing the corresponding factor fused to LexA. In each case, expression of the fusion protein restored the formation of the high-molecular-weight complex, and addition of anti-LexA antibodies resulted in a supershift compared to the wild type. These experiments demonstrate that Met28p and Met4p are capable of binding to the *MET16* upstream region in a high-molecular-weight complex together with Cbf1p. This result was confirmed by reconstitution experiments with purified recombinant Met28p in combination with various cell extracts. Addition of purified recombinant Met28p to an extract prepared from cells bearing a chromosomal deletion of the *MET28* gene supports the formation of a low-electrophoretic-mobility complex, which resembles that identified with wild-type extracts. In contrast, when the purified recombinant Met28p is used in combination with an extract prepared from cells that do not express either Met4p or Cbf1p, the low-mobility complex is not formed (135).

To determine which binary protein-protein contacts allow assembly of the Cbf1p-Met4p-Met28p complex, two-hybrid studies were carried out. Met4p and Met28p were thus shown to interact through their respective leucine zipper domains, while Met4p and Cbf1p were shown to be in contact through the bZIP domain of Met4p and the bHLH domain of Cbf1p. No direct interaction between Met28p and Cbf1p could be detected by such a method (135) (Fig. 11).

In Vitro Reconstitution of the Cbf1p-Met4p-Met28p Complex

To determine whether the Cbf1p-Met4p-Met28p complex could be assembled without additional factors, different recombinant derivatives of Cbf1p, Met4p, and Met28p were used. Each derivative was expressed in *E. coli* as glutathione transferase or polyhistidine tag fusions and subsequently purified by affinity chromatography (134). Each of the purified derivatives contained the domain which was shown to be in-

FIG. 10. The *MET16* promoter region protected by the Cbf1p-Met4p-Met28p complex as revealed by DNase I footprint experiments. The open box indicates protection by Cbf1 alone, and the dark grey box indicates protection by the Cbf1p-Met4p-Met28p complex. The TCACGTG core sequence present at position −175 in the *MET16* 5′ upstream region is shown as a solid bar (134).

volved in protein-protein interaction by the two-hybrid studies. The capacity of the recombinant proteins to form the complex was assayed in mobility shift experiments with an oligonucleotide probe corresponding to the *MET16* TCACGTG sequence and its adjacent nucleotides. These experiments demonstrated that addition of the three recombinant factors together with the DNA probe leads to the formation of two protein-DNA complexes, one containing Cbf1p alone and the other containing Cbf1p, Met4p, and Met28p complexed on the DNA probe. In contrast, the incubation of large amounts of Met4p, Met28p, or Met4p plus Met28p with the DNA probe in the absence of Cbf1p does not lead to formation of a protein-DNA complex, demonstrating Cbf1p dependence. DNase I footprint experiments performed with purified recombinant factors demonstrated that the Cbf1p-Met4p-Met28p complex forms over the TCACGTG sequence. Comparison of the footprints obtained with Cbf1p alone and with the three factors showed that assembly of the complex extends the protected region mainly toward the 5′ nucleotides (Fig. 10) (134).

The description of the transcription activation complex Cbf1p-Met4p-Met28p reinforces the emerging view of the regulation of gene expression in yeast. Transcription activation appears to be driven not only through the binding of isolated factors on upstream activating sequences but also by the assembly of highly specific multicomponent complexes. Apart from the Cbf1p-Met4p-Met28p complex, two other transcription activation complexes were demonstrated previously in *S. cerevisiae*, the HAP2-HAP3-HAP4-HAP5 (169) and the Swi4-Swi6 complexes (209). The Cbf1p-Met4p-Met28p complex exhibits several distinctive features. It associates one multifunctional factor (Cbf1p) with two specific factors (Met4p and Met28p). To our knowledge, this complex also constitutes the first description in eucaryotic cells of a complex associating one bHLH factor with two bZIP factors. Furthermore, the Cbf1p-Met4p-Met28p complex contains only one transcription activation module, provided by the Met4p subunit.

Enhancement of the Cbf1p-Met4p-Met28p Complex Assembly through Met28p Stimulation of Cbf1p DNA Binding Activity

The in vitro reconstitution experiments described in the preceding section revealed that Met28p is endowed with a specific function. As previously demonstrated, the recombinant Cbf1p is capable of binding to the *MET16* TCACGTG sequence in the absence of Met4p and Met28p (189). However, in mobility shift assays, when the binding reactions were performed at Cbf1p concentrations that result in very small amounts of Cbf1p-DNA complexes, the addition of Met28p to the reaction mixtures strongly increased the DNA binding activity of Cbf1p (134). This stimulation of the Cbf1p DNA binding activity is observed without changes in the mobility of the Cbf1-DNA complex. Such a result could be accounted for by the initial formation of Cbf1p-Met28p-DNA ternary complexes from which Met28p dissociates during electrophoresis. At least two examples of similar observations exist. (i) The homeodomain protein Phox from mammalian cells specifically increases binding of the serum response factor to its DNA binding site without visualization of the ternary Phox-serum response factor-DNA complex (86). (ii) Ternary complexes were not observed in the shift assays, demonstrating that the viral protein Tax enhances the DNA binding activity of several bZIP factors (259).

The stimulatory effect exerted by Met28p on Cbf1p DNA binding activity was further demonstrated through DNase I footprint assays. At low concentrations of Cbf1p, footprints over the TCACGTG sequence were observed only in the presence of Met28p. The protected region is essentially identical to that observed when large amounts of Cbf1p are added to the DNA binding reaction mixtures in the absence of Met28p. Furthermore, in both mobility shift assays and DNase I protection experiments, Met28p exerts its stimulatory effect on the binding of the Cbf1p-Met4p-Met28p complex to the *MET16* upstream activation sequence. Additional experiments were performed to determine how Met28p enhances the binding of Cbf1p to the DNA. Cbf1p, like other bHLH proteins, binds to DNA as a dimer. Since the Cbf1p dimers are extremely stable in solution (62), it is unlikely that Met28p exerts its stimulatory effects by increasing the dimerization rate. Association and dissociation rates of Cbf1p-DNA complexes were thus measured in the presence and absence of Met28p. These experiments revealed that part of the Met28p stimulation of Cbf1p-DNA binding was a decrease in the dissociation rates of the Cbf1p-DNA complexes in the presence of Met28p (134).

It appears that Met28p contributes to the transcriptional regulation of the sulfur amino acid pathway by performing two functions. First, it is an indispensable architectural component of the Cbf1p-Met4p-Met28p complex, and the interactions between its leucine zipper domain and that of Met4p are critical for the formation of this complex. Second, its stimulation of the DNA binding activity of Cbf1p increases the DNA binding affinity of the complex.

AdoMet-Mediated Regulation of Met4p Transcription Activation Function

The first insights into the Cbf1p-Met4p-Met28p complex-mediated transcription were provided by functional analysis of Met4p (136). As mentioned above, Met4p is a strong transcriptional activator. Expression of a LexA-Met4p fusion protein results in a high level of reporter gene expression, which decreases fourfold when the cells are grown under repressive conditions. Since the LexA-Met4p fusion proteins are brought to DNA by their LexA moiety, this finding suggests that under repressive growth conditions, the transcriptional activation function of Met4p is inhibited. To determine the molecular basis of this regulation, Met4p internal deletions were analyzed (136). About 60 different LexA-Met4p derivatives were constructed and expressed in yeast. These derivatives were tested for positive *lacZ* regulation when the cells were grown under either nonrepressive or repressive conditions.

The activation domain of Met4p was localized on its amino-terminal portion, between the amino acids 95 and 144. This activation domain is unique within Met4p and appears to have a biased composition, being rich in acidic residues as well as in asparagine residues. However, Met4p is a hydrophilic protein with a high content of negatively charged amino acids, and asparagine is its most common amino acid. It is thus doubtful that such a composition bias explains the function of this region. More likely, the transcription activation function of this domain is explained by the folding of this domain into a particular structure. As reported previously for the transcription

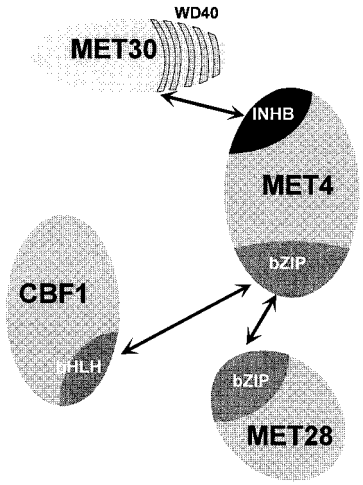

FIG. 11. Interactions between the different factors involved in regulation of the expression of the *MET* genes. Deduced from references 134 and 135.

activation domain of Gcn4p (95), progressive deletions within the Met4p activation domain lead to a gradual decrease of the transcription activation function, suggesting that the activation domain of Met4p contained independently functional modules. The Met4p activation domain was, moreover, shown to function in a constitutive manner. Under repressive conditions, the inhibition of the Met4p transcription activation function required a distinct domain of Met4p, located between residues 189 and 235, the inhibitory region. The use of LexA-Gal4p-Met4p hybrid proteins demonstrated that the Met4p inhibitory region is capable of controlling the function of the unrelated Gal4p activation domain under repressive conditions. An additional functional domain within Met4p, located between residues 312 and 375 of Met4p, is required for the maximal activity of Met4p under nonrepressive conditions. This domain, the auxiliary domain, is not a transcriptional activation domain but is necessary for relieving the function of the inhibitory region under nonrepressive conditions. All these results led to the following model to explain the AdoMet regulation of Met4p activity (Fig. 12). At high intracellular AdoMet concentrations, the inhibitory region interacts with a regulatory protein, which prevents the activation domain of Met4p from contacting the basal transcription apparatus, thereby resulting in low levels of transcription activation. At low AdoMet concentrations, the auxiliary domain helps the inhibitory region to dissociate from the regulatory protein, thereby freeing the activation domain to function (136). Omura et al. isolated two single-point mutations within Met4p that lead to constitutive expression of a *MET25-PHO5* gene fusion in cells grown under repressive conditions (192). These mutations result from a phenylalanine-to-serine substitution at residue 156 and from a serine-to-proline substitution at residue 215. The position of the second mutation is in a good agreement with the functional model proposed above, since residue 215 lies within the inhibitory region but residue 156 falls in an intermediary position, between the activation domain and the inhibitory region. This suggests that the former domain may be larger than was previously predicted. However, the Ser215Pro Met4p derivative exhibits a stronger phenotype than the Phe156Ser Met4p derivative and appears to be the only mutation resulting in a constitutive expression of the *MET25* gene when it was expressed in the presence of wild-type Met4p (192). This is an example of a dominant phenotype expected from a mutation that impairs the negative regulation of a transcriptional activator.

MET30 Encodes a Transcriptional Inhibitor of Met4p

The model depicted in the preceding section for the regulation of Met4p function postulates that a protein would specifically inhibit Met4p activity when the cells are grown under repressive conditions. The gene encoding this protein was identified by a specific genetic screen based on a *MET25-xylE* gene fusion. The *xylE* gene from *Pseudomonas putida* encodes a catechol oxidase (263), whose activity can be visualized in colonies sprayed with catechol. Yeast cells containing XylEp under the control of the *MET25* promoter turn yellow when grown under nonrepressive conditions but remain white under repressive condition (102). The *MET30* locus was identified by a mutation that leads to yellow cells under repressive growth conditions (249). Enzymatic analyses performed with a *met30* mutant showed impaired AdoMet-mediated repression of the sulfate assimilation pathway. The results were confirmed by Northern blot analyses. *MET30* encodes a protein of 640 residues, which is devoid of canonical DNA binding motifs but contains five 40-amino-acid WD40 motifs in its carboxy-terminal region (249). Since this motif was first identified as the major part of the trimeric G-protein β-subunit, it is also known as the β-transducing motif (186).

Met30p function was studied with various LexA-Met4 fusion proteins expressed in wild-type and *met30* mutant cells. When the fusion proteins were expressed in *met30* mutants, inhibition of Met4p-mediated transcription was not detected in cells grown under repressive conditions. These results were consistent with Met30p inhibiting Met4p at high intracellular AdoMet concentrations. This hypothesis was supported by two-hybrid experiments where Met4p and Met30p were shown to interact in vivo. Furthermore, the Met30p-Met4p interaction is significantly decreased by deletion of the Met4p inhibitory region (249). The Met30p region involved in the interaction with Met4p lies in the most amino-terminal WD40 motif (138). Thus, Met30p exhibits the functional characteristics of the regulatory protein predicted by the model proposed for the AdoMet-mediated regulation of Met4p activity (Fig. 12).

Surprisingly, Met30p function is not restricted to regulation of sulfate assimilation. *met30* mutants constitutively express *MET19*, which encodes glucose-6-phosphate dehydrogenase (247, 249). This is consistent with Met30p regulating the activity of transcriptional activators unrelated to Met4p, because *MET19* transcription is Met4p independent (253). That Met30p contains several WD40 motifs might explain how it could interact with different activators, since each of these motifs has the potential of interacting with one protein, as demonstrated by Komachi et al. during analyses of interactions between Tup1p and the homeodomain protein a2 (123).

Bai et al. have recently identified another functional domain within Met30p between residues 187 and 250 (6). This region is similar to a structural motif, the F-box, found in a family of eucaryotic proteins including the yeast proteins cyclin F, Cdc4p, Skp2p, Ggr1p, and Met30p. This motif was required for Cdc4p and Skp2p to interact with Skp1p, a component of the multiprotein Cbf3 complex that binds to the CDEIII element of yeast centromeres (6, 52). Met30p indeed interacts with Skp1p through its F-box motif (138). It was hypothesized that such interactions may allow Skp1p to target the F-box protein to the ubiquitin proteolytic pathway (6). This hypothesis was further supported by the fact that Met30p interacts with Cdc53p, a component of ubiquitin-protein ligase (255a). It is therefore possible that the Met4p regulation by Met30p in-

volves proteolysis of Met4p. However, it must be recalled that LexA-Met4p fusion proteins were demonstrated by Western blots to be present in equivalent amounts whether cells were grown under nonrepressive or repressive conditions (136). Whether Skp1p may be found, like Cbf1p, in chromosomal sites other than centromeres has not been established. Whether interactions between two architectural components of yeast centromeres and two regulatory factors of the sulfur amino acid metabolism are biologically significant remains an open question. In this regard, Met30p was found to be essential, although no explanation for its necessity is available (249).

Regulatory Loop within the Sulfur Network

The results reported above have shed light on the regulation of the function of several *trans*-acting regulatory factors. In addition, other studies have addressed the question of how the regulatory genes themselves are regulated. To our knowledge, no regulation specifically affecting the expression of *CBF1* has been reported. The transcription of both *CBF1* and *MET4* was found to be independent of the intracellular level of AdoMet (10). However, *MET4* expression is subject, to a certain extent, to general amino acid control: Mountain et al. reported that the presence of 3-aminotriazole (3-AT) in the medium resulted in an increased *MET4* transcription (175), although this 3-AT response was not as strong as that measured for the *HIS4* gene, a well-established target of Gcn4p. However, contrary to what is observed for *HIS4*, the level of *MET4* transcripts in the presence of 3-AT is equivalent in wild-type and *gcn4* mutant cells (175).

In contrast, transcription of the *MET28* gene was demonstrated to be regulated by increases in intracellular levels of AdoMet (134). Northern blot experiments revealed that the addition of 1 mM L-methionine to the growth medium resulted in a rapid cessation of *MET28* transcription. The *MET28* transcripts were shown to be very unstable, having a half-life of less than 5 min. Additional Northern blot experiments revealed that *MET28* transcription depends on the presence of functional Met4p. It was further demonstrated that both Cbf1p and Met28p are involved in the transcriptional activation of the *MET28* gene. The biochemical activity of Met28p, which allows it to stimulate the Cbf1p DNA binding activity, and the mechanisms underlying the transcriptional control of the *MET28* gene demonstrate the existence of a positive regulatory loop within the sulfur network (Fig. 11) (134).

Met31p and Met32p, Two New Regulatory Factors

As mentioned above, analysis of the *MET25* upstream region pointed to the existence of another *cis*-acting element in addition to the TCACGTG sequence. Deletion of nucleotides around position −200 impaired the repression of *MET25* transcription twofold (246). The −200 region of *MET25* contains a short sequence, AAANTGTG, which is found in almost all the *MET* genes (Fig. 9), suggesting that it might be a regulatory element. To analyze the potential function of this element, specific DNA binding proteins recognizing this sequence were isolated by the one-hybrid method (17). *MET31*, encoding a small zinc finger-containing protein, was identified in this way (17). Met31p is similar to the product of another gene, *MET32*, identified by a mutation that alters methionine uptake. Met31p and Met32p are 46% identical, and both possess two zinc finger domains: an amino-terminal proximal CC/HH type and a carboxy-terminal CC/HC type. Recombinant Met31p and Met32p were both shown to recognize the AAANTGTG sequence by gel mobility shift assays and DNase I footprint assays. Unexpectedly, both Met31p and Met32p were found to be capable of transcriptional activation in the LexA assay system, in a Met4p-dependent manner. Northern blot analysis of cells that do not express Met31p and/or Met32p shows that the function of the two proteins during transcription of the sulfur genes varies from one gene to the other: the expression of *MET3* and *MET14* depends strictly on the presence of both Met31p and Met32p while that of *MET25* is constitutive in cells lacking both Met31p and Met32p. This latter result may explain why the AAANTGTG DNA element was first identified as a negative *cis*-acting element during mutational analysis of the *MET25* upstream region. Results obtained so far argue that the roles of Met31p and Met32p vary from one gene to the next.

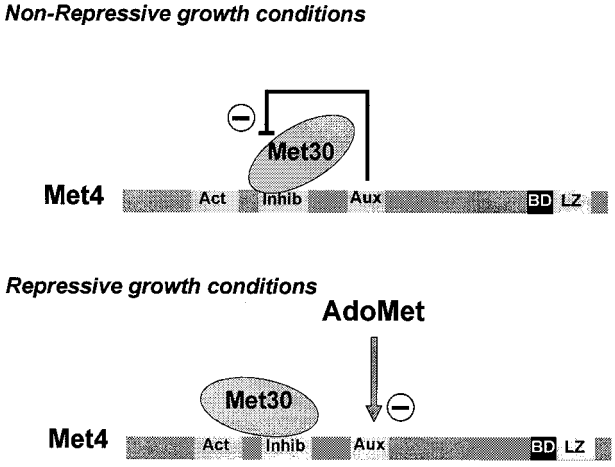

FIG. 12. Model for the AdoMet regulation of Met4p activation function. The model is deduced from the results described in references 136 and 249. Act, activation domain; Inhib, inhibitory region; Δux, auxiliary domain; BD, basic domain; LZ, leucine zipper.

Regulation of Sulfur Metabolism in Other Fungi

Sulfur amino acid metabolism has not been well studied in other fungi; insights have been derived mainly from *N. crassa*. These studies focused on the regulation of genes encoding catabolic enzymes responsible for the use of secondary sulfur sources, such as choline-*O*-sulfate or aromatic sulfate (for a review, see reference 165). Genetic studies have identified three genes encoding sulfur *trans*-acting regulators, one positive (*Cys3*) and two negative (*Scon1* and *Scon2*) (22). Only the *Cys3* and *Scon2* genes have been cloned. Cys3p consists of 236 amino acids and, like the yeast Met4p and Met28p, is a bZIP protein (75). The Cys3p bZIP region exhibits significant sequence similarity to both Met4p and Met28p. Met4p and Cys3p have similar leucine zippers, and the Met28p basic region is similar to that of Cys3p (135). Unlike Met4p and Met28p, Cys3p binds by itself to the upstream regions of coregulated genes. Cys3p binding sites show limited sequence similarities, and mutational analysis has recently defined the consensus Cys3p binding sequence to be 5′-ATGPuPyPuPyCAT-3′ (150). The N-terminal region of Cys3p contains regions rich in proline and ones rich in acidic amino acids, which are essential for the function of Cys3p (for a review, see reference 165). The proline-rich region appears to represent the activation domain of Cys3p. The second sulfur-regulatory factor studied in *N. crassa* is the negative regulator Scon2p. *Scon2* mutants constitutively express the sulfur catabolic enzymes. Scon2p possesses high sequence similarity to Met30p (133). Like Met30p, Scon2p contains WD40 repeats in its carboxy-

terminal region and also contains the F-Box identified by Bai et al. (6). It is thus reasonable to imagine that Scon2p may be a functional homolog of Met30p in *N. crassa*, although how Scon2p acts in the negative regulation of sulfur metabolism is unknown. *Scon2* transcription is regulated by Cys3p (133).

The pathogenic fungus *Histoplasma capsulatum* has the capacity to grow in either a yeastlike or a mycelial form in response to environmental stimuli. This phase transition is reversible and can be easily reproduced under laboratory conditions by shifting the cells from 23 to 37°C. Cysteine is needed in different steps of the mycelium-to-yeast-phase transition, suggesting a cell cycle control of the biosynthesis of cysteine during the transition (162).

Variations in Regulatory Mechanisms

Although many aspects of the AdoMet regulatory circuits remain to be deciphered, the genetic and molecular approaches have illuminated several aspects of the mechanisms underlying this regulation. The most unexpected trait revealed by these studies is that the mechanisms vary from one coregulated gene to another. It had been anticipated that the coregulation of a set of unlinked genes might arise from the binding of the same array of transcriptional activators to DNA elements present in each of these genes. According to such a principle, several studies devoted to the regulation of gene expression tend to focus on one particular and well-established gene taken as a relevant model for the entire set of the network it belongs to. In the case of the AdoMet-mediated regulation of the sulfur amino acid metabolism, both in vivo and in vitro experiments have clearly demonstrated that such an assumption should be carefully examined. Indeed, Northern blot analysis has revealed that the effect of the mutation of each *trans*-acting factor on transcriptional activation may vary from one structural gene to the next (137). Moreover, in vitro experiments have led to the conclusion that the Cbf1p-Met4p-Met28p complex may not assemble in the same way on all of the 5′ upstream regions of the *MET* genes. For example, the Cbf1p-Met4p-Met28p complex could be formed by recombinant proteins on the *MET16* promoter but did not form on either the *MET25* or the *MET28* upstream region (134, 135, 138). In this regard, transcriptional activation of *MET16* is strictly dependent on a functional Cbf1p, whereas the *MET25* and *MET28* genes are two of the *MET* genes whose transcription is only weakly decreased by a *CBF1* chromosomal deletion (135, 137). Taken together, these in vitro and in vivo data suggest that for some of the *MET* genes, there may be another yeast factor that, in addition to Cbf1p, participates in the tethering of Met4p to the promoter regions. The molecular variations of the mechanisms leading to the AdoMet-mediated regulation of the sulfur network were further emphasized by the recent analysis of Met31p and Met32p, which appear not to act during the transcriptional regulation of all the *MET* genes (17). It is therefore possible that such mechanistic variations in specific regulation represent a general feature of regulatory mechanisms in eucaryotic cells.

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