Cysteine Biosynthesis in Saccharomyces cerevisiae Occurs through the Transsulfuration Pathway Which Has Been Built Up by Enzyme Recruitment

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The transsulfuration pathways allow the interconversion of homocysteine and cysteine with the intermediary formation of cystathionine. The various organisms studied up to now incorporate reduced sulfur into a threeor a four-carbon chain and use differently the transsulfuration pathways to synthesize sulfur amino acids. In enteric bacteria, the synthesis of cysteine is the first step of organic sulfur metabolism and homocysteine is derived from cysteine. Fungi are capable of incorporating reduced sulfur into a four-carbon chain, and they possess two operating transsulfuration pathways. By contrast, synthesis of cysteine from homocysteine is the only existing transsulfuration pathway in mammals. In *Saccharomyces cerevisiae*, genetic, phenotypic, and enzymatic study of mutants has allowed us to demonstrate that homocysteine is the first sulfur amino acid to be synthesized and cysteine is derived only from homocysteine (H. Cherest and Y. Surdin-Kerjan, Genetics 130:51–58, 1992). We report here the cloning of genes *STR4* and *STR1*, encoding cystathionine β -synthase and cystathionine γ -lyase, respectively. The only phenotypic consequence of the inactivation of *STR1* or *STR4* is cysteine auxotrophy. The sequencing of gene *STR4* has allowed us to compare all of the known sequences of transsulfuration enzymes and enzymes catalyzing the incorporation of reduced sulfur in carbon chains. These comparisons reveal a partition into two families based on sequence motifs. This partition mainly correlates with similarities in the catalytic mechanisms of these enzymes.

The increasing knowledge of related biosynthetic pathways common to numerous organisms emphasizes the prevalent occurrence of metabolic diversity. Molecular studies of metabolic diversity now offer a propitious basis for insight into the evolutionary mechanisms that allowed acquisition of multistep pathways. Two major working hypotheses have been proposed to describe the establishment of such pathways. The first one (12) invokes a retrieval recruitment of new enzymes progressively built backwards from the final metabolite of the pathway. The second one (15, 38) suggests that primitive enzymes possessed a very broad specificity permitting subsequent elaboration of new enzymes following gene duplication. The latter hypothesis has received some support from the observed sequence similarities between enzymes catalyzing consecutive steps in the isoleucine biosynthetic pathway (25).

Transsulfuration metabolism is a well-documented case of a pathway exhibiting alternative means whereby various organisms synthesize their metabolites. The transsulfuration pathways allow the interconversion of homocysteine and cysteine with the intermediary formation of cystathionine (Fig. 1). Enteric bacteria derive the sulfur moiety of homocysteine solely from cysteine. Fungi are capable of incorporating reduced sulfur into a four-carbon chain, and they possess two operating transsulfuration pathways. By contrast, synthesis of cysteine from homocysteine is the only existing transsulfuration pathway in mammals (Fig. 1). Moreover, alternative carbon substrates are used for these syntheses, depending on the organism: the four-carbon ester involved in homocysteine synthesis could be either O-succinylhomoserine, O-acetylhomoserine, or phosphohomoIn addition to the biochemical diversity of transsulfuration metabolism among extant species, it is well known that in vitro, transsulfuration enzymes exhibit broad specificities. For example, *Escherichia coli* cysteine synthase and yeast homocysteine synthase are both capable of catalyzing various reactions: the former can use triazole as a substrate (18), while the latter can incorporate reduced sulfur into a threecarbon chain (37). Cystathionine γ -synthase and cystathionine β -lyase both can use cystine as a substrate. Deamination of L-serine may also be carried out by cystathionine β -lyase (3). It must be noted that all of the transsulfuration enzymes utilize pyridoxal phosphate as a common cofactor.

Fungi appear to be the organisms which retain the most complex transsulfuration metabolism (Fig. 1). Genetic, phenotypic, and enzymatic study of *Saccharomyces cerevisiae* mutants allowed us to demonstrate that in yeast cells, cysteine derives only from homocysteine (5). Acquisition of molecular data on the yeast transsulfuration metabolism thus seems propitious for defining evolutionary relationships between related proteins that no longer serve the same function.

We report here the cloning and sequencing of gene STR4 as well as the study of gene STR1, encoding cystathionine β -synthase and cystathionine γ -lyase, respectively. Phenotypic consequences of the inactivation of each gene are described. Comparisons of all known sequences of transsulfuration enzymes reveals a partition into two families based on sequence motifs. This partition mainly correlates with similarities in the catalytic mechanisms of these enzymes.

serine (for a review, see reference 37). Likewise, the threecarbon chain utilized for cysteine synthesis can be esterified or not (36).

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FIG. 1. Existing transsulfuration pathways in S. cerevisiae (A), mammals (B), and E. coli (C). Enzymes: 1, homocysteine synthase; 2, cystathionine β -synthase; 3, cystathionine γ -lyase; 4, cysteine synthase; 5, cystathionine γ -synthase; 6, cystathionine β -lyase.

MATERIALS AND METHODS

Strains, media, and microbiological techniques. E. coli HB101 and JM103 were used as hosts for plasmid maintenance. S. cerevisiae strains used in this work are listed in Table 1. To grow E. coli, we used media described by Maniatis et al. (21). For S. cerevisiae, YPG and YNBG media were as described previously (5). Glutathione was used in some experiments as a source of cysteine (8). According to the auxotrophic requirements of strains, uracil (20 μ g/ml), adenine (40 μ g/ml), histidine (200 μ g/ml), leucine (100 μ g/ml), and tryptophan (20 μ g/ml) were added to the growth media. E. coli was transformed as described by Cohen et al. (7). S. cerevisiae was transformed after lithium chloride treatment as described by Ito et al. (14). Genetic crosses, sporulation, dissection, and scoring of nutritional markers were done as described by Sherman et al. (30).

Plasmid vectors. The multicopy plasmid pEMBLYe23 and the integrative plasmid pEMBLYi22, both bearing gene URA3 as a selectable marker, were used (1). The S. cerevisiae genomic library used for the cloning of genes STR1 and STR4 was previously described (35).

Plasmid integration and genetic analysis. To show that the insert present in plasmid pSTR4-2 was able to direct plasmid integration to a site on the genome linked to gene STR4, we constructed the following plasmid. The sequences from the 2μ m plasmid were deleted from plasmid pSTR4-2, yielding plasmid pSTR4-01. Plasmid pSTR4-01 was linearized by XbaI to direct the integration to the homologous genomic sequences and used to transform strain W303-1A (*ura3 STR4*). One resulting Ura⁺ transformant was crossed to

TABLE 1. Strains used

Strain	Genotype	Source
W303-1A	MATa ade2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1	R. Rothstein
W303-1B	MAT a ade2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1	R. Rothstein
CC627-2B	MATα his3 ura3 str4-1	This work
CC645-23C	MATa trp1-1 ura3 str4-1	This work
CC639-7D	MATa leu2 ura3 str1-1	This work
CC659-4B	MATa his3 ura3 str1-1	This work
C180	MATa ade2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 str4::URA3	This work
C181	MATa ade2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 str1::URA3	This work

strain CC645-23C (*ura3 str4-1*). The diploid was sporulated, and its meiotic progeny was analyzed in 20 tetrads. In 19 tetrads, all auxotrophic characters segregated perfectly $2^+/2^-$, and glutathione-positive (Glt⁺) spores were all Ura⁺, showing genetic linkage between the mutant locus and the integrated cloned DNA. One tetrad segregated $3^+/1^-$ for the glutathione auxotrophy, and one of the Glt⁺ spores was Ura⁻. This can be accounted for by the high frequency of gene conversion evidenced at the *STR4* locus (5).

To show that the insert present in plasmid pSTR1-1 was able to direct plasmid integration to a site on the genome linked to gene STR1, we constructed the following plasmid. The HindIII-XhoI fragment of plasmid pSTR1-1 was inserted in the HindIII and SalI sites of plasmid pEMBLYi22. The resulting plasmid, pSTR1-01, was linearized by SalI and was used to transform strain W303-1A (ura3 STR1) to uracil prototrophy. One resulting Ura+ transformant was crossed to strain CC659-4B (ura3 str1-1). The diploid was sporulated, and its meiotic progeny was analyzed in 18 tetrads. In 16 tetrads, a perfect $2^+/2^-$ segregation was observed for all characters, and all Glt⁺ spores were Ura⁺, showing genetic linkage between the mutant locus and the cloned DNA. Two tetrads exhibited a $3^+/1^-$ segregation for the glutathione auxotrophy, and in each tetrad one Glt⁺ spore was Ura⁻. Again, this results from the high frequency of meiotic gene conversion reported for the STR1 locus (5).

Recombinant DNA methods. Plasmid purification was performed as described by Ish-Horowicz and Burke (13). Genomic DNA analysis was performed with DNA prepared as described by Hoffman and Winston (11). Probes were made radioactive by the random-priming method described by Hodgson and Fisk (10).

Systematic deletion subclones were generated by the method described by Thomas and Surdin-Kerjan (34). Single-stranded phage DNA prepared from these deletions was sequenced by using the Pharmacia T7 sequencing kit. Analysis of the DNA sequence and comparisons on a VAX computer were made possible by the computer facilities of CITI2 in Paris.

Enzymatic assays. Cystathionine β -synthase and cystathionine γ -lyase were assayed as described previously (5). Protein concentrations were estimated by the method described by Lowry et al. (20).

Nucleotide sequence accession number. The nucleotide sequence of gene *STR4* reported here has been assigned EMBL accession number X72922.

RESULTS

Cloning and sequencing of the cystathionine B-synthaseencoding gene from S. cerevisiae. The cystathionine β -synthase gene (STR4) from S. cerevisiae was cloned by complementation of the glutathione auxotrophy of strain CC627-2B. The strain was transformed with the gene library described in Materials and Methods. Among 18,000 Ura⁺ transformants tested, three strains were found to grow without glutathione. Two clones harbored identical plasmids with a 7-kbp insert. The third one harbored a plasmid with a 8.7-kbp insert partly overlapping the first insert. The 7-kbp insert was subcloned, and the sequences required to complement the str4 mutation of strain CC627-2B were mapped to the HindIII-SphI fragment (Fig. 2). To ascertain that gene STR4 was cloned, we verified that the DNA sequences of the insert of plasmid pSTR4-2 were able to direct the integration of the plasmid to a site linked to gene STR4 on the genome (see Materials and Methods).



FIG. 2. Physical maps of the STR1 and STR4 regions. Fragments subcloned in plasmid pEMBLYe23 as well as their abilities to complement the str1 mutation (strain CC639-7D) or the str4 mutation (strain CC627-2B) are shown. ORF, open reading frame.

Cystathionine β -synthase was assayed in strain CC627-2B (*str4*) transformed with plasmid pSTR4-1. In extracts of such a strain, a 10-fold increase of cystathionine β -synthase activity over that of the parental wild-type strain was measured, in agreement with cystathionine β -synthase structural gene being expressed from a multicopy plasmid (Table 2).

The *Hin*dIII-*Sph*I fragment of plasmid pSTR4-2 was sequenced as described in Materials and Methods. The sequence was entirely determined on both strands. The nucleotide sequence and the polypeptide sequence deduced from the longest open reading frame are shown in Fig. 3.

Cloning of the cystathionine γ -lyase-encoding gene from S. cerevisiae. The cystathionine γ -lyase gene (STR1) was cloned by complementation of the glutathione auxotrophy of strain CC639-7D. The strain was transformed with the gene library

TABLE 2. Enzyme activities of different strains

Strain	Relevant	Plasmid	Enzyme activity (nmol of substrate transformed/min/mg of protein)				
	genotype	present	Cystathionine β-synthase	Cystathionine γ-lyase			
W303-1B			30	9.6			
CC627-2B	str4		0	7.2			
CC627-2B	str4	pSTR4-1	377	8.8			
CC639-7D	str1	•	38	<1			
CC639-7D	str1	pSTR1-1	42	178			

described in Materials and Methods. Among 16,000 Ura⁺ transformants tested, six strains were found to grow without glutathione. These six strains harbored the same plasmid with a 6.6-kbp insert. By subcloning, the sequences able to complement the glutathione auxotrophy of strain CC639-7D were mapped to the 3.4-kbp *Hin*dIII-*Xho*I fragment (Fig. 2). To ascertain that gene *STR1* was cloned, we verified that the DNA sequences of the insert of plasmid pSTR1-1 were able to direct the integration of the plasmid to a site linked to gene *STR1* on the genome (see Materials and Methods).

Cystathionine γ -lyase was assayed in strain CC639-7D (*str1*) transformed with plasmid pSTR1-1. In extracts of such a strain, a 17-fold increase of cystathionine γ -lyase activity over that of the parental wild-type strain was measured, in agreement with cystathionine γ -lyase structural gene being expressed from a multicopy plasmid (Table 2).

While this work was being carried out, Ono et al. (24) reported the cloning and sequencing of the CYS3 gene from S. cerevisiae. The restriction map of this gene is identical with that of STR1 reported here. This finding confirms that the two loci represent the same gene, the conclusion previously reached by our genetic analysis (5).

Gene disruption alleles at either STR1 or STR4 result in cysteine auxotroph yeast strains. Gene disruption alleles at STR1 and STR4 were constructed as described by Rothstein (27). To disrupt gene STR1, the 1.1-kbp StuI fragment of plasmid pSTR1-01 (described in Materials and Methods) was replaced by the URA3 gene. The resulting plasmid was cut by HindIII and used to transform strain W303-1A to uracil

aagcttcagttgcattctaaccttatcacaacaacttcaacttcacccaagtaaggataatcag									-289									
ctctgtcgtgactgataaatgctatatccggcatatgcagtccacacggcattaccgtttcactaatttatt									-217									
ttaa	agta	aaco	rcaao	racad	ttca	ccad	actt	gtat	atat	aato	atcat	gato	ctto	tato	rccaa	agta	laaag	-73
gcaacacttgaagatttcgttgtaggccacttgctcaaaggacatctagataaatacgacgtaagaataaaa -								-1										
ATG	ACT	ААА	тст	GAG	CAG	CAA	GCC	GAT	TCA	AGA	CAT	AAC	GTT	ATC	GAC	TTA	GTT	54
М	Т	K	S	Е	Q	Q	A	D	S	R	н	N	v	I	D	L	v	
GGT	AAC	ACC	CCA	TTG	ATC	GCA	CTG	AAA	AAA	TTG	CCT	AAG	GCT	TTG	GGT	ATC	AAA	108
G	N CAA	Т	Р тат	L	1	A	L	CTA	K TAC	ይ እእጥ	CCA	K CCT	A	TCC	ATC	1	GAC	162
P	Q	Ĩ	Ŷ	A	K	L	E	L	Ŷ	N	P	G	G	ŝ	ĩ	K	D	102
AGA	ATT	GCC	AAG	TCT	ATG	GTG	GĀA	GĀA	GCT	GAA	GCT	TCC	GGT	AGA	ATT	CAT	CCT	216
R	I	A	K	S	M	V CNN	E	E	A	E	A	S	G	R	I	H	P	270
s	R	s	T	L	I	E	P	T	S	G	N	T	G	I	G	L	A	210
TTA	ATC	GGC	GCC	ATC	AAA	GGT	TAC	AGA	ACT	ATC	ATC	ACC	TTG	CCG	GAA	AAA	ATG	324
L	I	G	A	I	K	G	Y	R	T	I	I	Т	L	P	E	K	M	270
TCT	AAC	GAG	K	GTT V	S	v	CTA L	AAG	GCT	CTG L	GGT	A	GAA E	T	T	R	T	3/8
CCA	ACT	GCT	GCT	GCC	TGG	GAT	TCT	CCA	GAA	TCA	CĂT	ATT	GGT	GTT	GCT	AAG	AAG	432
P	т	A	A	A	W	D	s	P	Е	s	H	I	G	v	A	K	к	
TTG	GAA	AAA	GAG	ATT	CCT	GGT	GCT	GTT	ATA	CTT	GAC	CAA	TAT	AAC	AAT	ATG	ATG	486
77C	E CCA	C A A	CCT.	1	TAC	G TTT	A	ACT	L	CGC	GAA	ATC	CAA	N AGA	CAG	CTA	GAA	540
N	P	E	A	H	Ŷ	F	G	T	G	R	E	I	Q	R	Q	L	E	040
GAC	TTG	AAT	TTA	TTT	GAT	AAT	CTA	CGC	GCT	GTT	GTT	GCT	GGT	GCT	GGT	ACT	GGT	594
D	L	N	L	F	D	N	L	R	A	v	V	A	G	A	G	T	G	C 4 0
GGG	ACT	ATT	AGC	GGT	ATT	TCC	AAG	TAC	TTG T.	AAA K	GAA	CAG	AAT	GAT	AAG	ATC	CAA	648
ATC	GTT	GGT	GCT	GAC	ĊĊA	TTC	GGT	TCA	ATT	TTA	GCC	CÃA	ССТ	GÃA	AAC	TTG	AĂT	702
I	v	G	A	D	Ρ	F	G	s	I	L	A	Q	P	Е	N	L	N	
AAG	ACT	GAT	ATC	ACT	GAC	TAC	AAA	GTT	GAG	GGT	ATT	GGT	TAT	GAT	TTT	GTT	CCT	756
CAG	GTT	TTG	GAC	AGA		ТТА	АТТ	GAT	GTT	тсс	TAT	AAG	ACA	GAC	GAC	AAG	сст	810
Q	v	L	D	R	ĸ	L	I	D	v	W	Ŷ	ĸ	Т	D	D	ĸ	P	
TCT	TTC	AAA	TAC	GCC	AGA	CAA	TTG	ATT	TCT	AAC	GAA	GGT	GTC	TTG	GTG	GGT	GGT	864
S	F	K	Y	A	R	2 2 2	L	I	S	N	E	G	CNN	L	CNC	G	G	010
S	s	GGI	S	A	F	T	A	V	v	K	Y	C	E	D	H	P	E	910
CTG	ACT	GĂA	GAT	GAT	GTC	ATT	GTT	GCC	ATA	TTC	CCA	GAT	тсс	ATC	AGG	TCG	TAC	972
L	Т	Е	D	D	V	I	v	A	I	F	Р	D	S	I	R	S	Y	
CTA	ACC	AAA	TTC	GTC	GAT	GAC	GAA	TGG w	TTG	AAA	AAG	AAC	AAT	TTG	TGG	GAT	GAT	1026
GAC	GTG	TTG	GCC	CGT	TTT	GAC	TCT	TCA	AAG	CTG	GAG	GCT	TCG	ACG	ACA	AAA	TAC	1080
D	v	L	A	R	F	D	S	S	K	L	E	A	S	Т	Т	K	Y	
GCT	GAT	GTG	TTT	GGT	AAC	GCT	ACT	GTA	AAG	GAT	CTT	CAC	TTG	AAA	CCG	GTT	GTT	1134
A	CTT	NAC	CNN CNN	ACC	N CCT	A	T GTC	ACT	CAT	CTT	ATC	H AAG	ር አጥል	K ጥጥል	2 2 2 2	GAC	V DDT	1188
S	v	K	E	T	A	K	v	T	D	v	I	K	I	L	K	D	N	1100
GGC	TTT	GAC	CAA	TTG	ССТ	GTG	TTG	ACT	GĀA	GAC	GGC	AAG	TTG	TCT	GGT	TTA	GTT	1242
G	F	D	Q	L	Р	V	L	Т	E	D	G	ĸ	L	S	G	L	V	
ACT	CTC	TCT	GAG	CTT	CTA	AGA	AAA	CTA	TCA	ATC	AAT	AAT	TCA	AAC	AAC	GAC		1296
ACT	ATA	AAG	GGT	AAA	TAC	TTG	GAC	TTC	AAG	AAA	TTA	AAC	AAT	TTC	AAT	GAT	GTT	1350
Т	I	K	G	К	Y	L	D	F	K	K	L	N	N	F	N	D	v	
TCC	TCT	TAC	AAC	GAA	AAT	AAA	TCC	GGT	AAG	AAG	AAG	TTT	ATT	AAA	TTC	GAT	GAA	1404
S	S	Y NAC	N	E	N	K	S NNTT	G	K	K	K CNN	F	I	K	F	D	E	1459
N	S	K	L	S	D	L	N	R	F	F	E	K	N	S	s	A	v	1450
ATC	ACT	GAT	GGC	TTG	AAA	CCA	ATC	CAT	ATC	GTT	ACT	AAG	ATG	GAT	TTA	CTG	AGC	1512
I	T	D	G	L	ĸ	P	I	н	I	v	т	K	м	D	L	L	S	
TAC	TTA	GCA	TAA *	ata	agaa	accca	acgct	tca	aataa	aaago	caaa	catao	gaago	caaaa	atcco	gtcat	tcct	1579
ttoctattocaccottotottatataactacttaattaaataccocctataccaacca										1651								
ctattatttttacaaattccttatcatcggatcc											1685							
	1				1			f tha	стр	1			ha 5'	and	2/ 8	onki		ione

FIG. 3. Nucleotide and deduced amino acid sequences of the STR4 gene with the 5' and 3' flanking regions. The noncoding strand is shown. The nucleotide sequence is numbered from nucleotide 1 of the presumed initiation codon.

prototrophy. DNA blot analyses of genomic DNA isolated from the parental strain W303-1A and from one of the transformed strains (C181) are shown in Fig. 4a. In strain C181, a 1.2-kbp fragment is detected as expected from the cleavage at the *Eco*RV site in the *URA3* sequence instead of the 3.9-kbp fragment revealed in the parental strain W303-1A. To disrupt gene *STR4*, the *Xba1-Eco*RI fragment of pSTR4-01 (described in Materials and Methods) was replaced by the *URA3* gene. The resulting plasmid was cut by *Hind*III and used to transform strain W303-1A to uracil prototrophy. DNA blot analyses of genomic DNA isolated from the parental strain W303-1A and from one of the transformed strains (C180) are shown in Fig. 4b. In the case of strain W303-1A, one band accounting for the two expected *Eco*RV fragments (2.1 and 2.0 kbp) is revealed. For the transformed strain C180, a 0.9-kbp fragment is detected, as expected from the cleavage at the EcoRV site in the URA3 sequence.

Strains C180 and C181 were both shown to require cysteine for growth. As expected, this nutritional requirement can be also satisfied by the addition of glutathione to the medium. In contrast, other organic sulfur compounds such as homocysteine, methionine, and S-adenosylmethionine do not sustain growth of these two strains. These results confirm that in S. cerevisiae, cysteine is derived from homocysteine only through the transsulfuration pathway. S. cerevisiae thus appears to be capable of incorporating inorganic reduced sulfur only into a four-carbon chain yielding homocysteine.

Transsulfuration enzymes are divided into two mutually exclusive classes. The sequences of cystathionine β -synthase and cystathionine γ -lyase from S. cerevisiae were compared

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FIG. 4. (a) Southern blot analysis of STR1 gene disruption. Lane A, EcoRV-HindIII digest of genomic DNA extracted from strain W303-1A; lane B, EcoRV-HindIII digest of genomic DNA extracted from strain C181. The filter was probed with the PstI-PstI fragment. (b) Southern blot analysis of the STR4 gene disruption. Lane C, EcoRV digest of genomic DNA extracted from strain W303-1A; lane D, EcoRV digest of genomic DNA extracted from strain C180. The filter was probed with the XbaI-SphI fragment.

against those in the National Biomedical Research Foundation sequence collection (version 74). Additional sequences were entered by us directly from the literature. Partial comparisons have already been published (9, 26, 28, 33). The sequences were aligned by using the Macaw program (29). Local alignments were optimized without creating additional gaps between the blocks of similarities. This search showed that transsulfuration enzymes can be partitioned into two exclusive families.

The first family is composed of the cystathionine β -synthases and cysteine synthases. Rat and yeast cystathionine β -synthases, 560 and 506 amino acid residues long, respectively, show extensive homology that encompasses the entire length of the yeast enzyme. The score of identity is 40% (199 identical residues). The rat enzyme has a 60-amino-acid long N-terminal extension, which accounts for the size difference between the two cystathionine β -synthases. These enzymes also exhibit extensive homology with the cysteine synthases from bacteria and plants (Fig. 5). For example, 120 amino acid residues are identical between the yeast cystathionine β -synthase and the cysteine synthase from spinach (37% identity with respect to the yeast enzyme), compared with the level of homology existing between the two cysteine synthases from *E. coli* (124 identical

residues) or with that existing between the cysteine synthase from spinach and its *E. coli* counterpart encoded by the *cysK* gene (158 identical residues). It must be stressed that 59 amino acid residues (20%) are strictly conserved between the five enzymes of this family.

The second family includes the rat and yeast cystathionine γ -lyases, the yeast homocysteine synthase, and both the cystathionine γ -synthase and cystathionine β -lyase from E. coli (Fig. 6). The overall alignment reveals the presence of nine significant blocks of similarities which are dispersed along the polypeptides. The rat and yeast cystathionine γ -lyases are 47% conserved (170 identical residues). The yeast cystathionine γ -lyase also shows a high level of homology with the *E. coli* cystathionine β -lyase (94 identical residues) as well as with the E. coli cystathionine γ -synthase (128 identical residues). Similar levels of homology exist between the yeast homocysteine synthase and other members of this class; for example, yeast homocysteine synthase shares 99 and 104 identical amino acid residues with the E. coli cystathionine y-synthase and the yeast cystathionine γ -lyase, respectively. However, the homocysteine synthase differs from the others by containing an insertion of about 30 amino acid residues in its middle part.

All of the transsulfuration enzymes utilize the same cofac-

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Rat-βSase ScStr4 Sp-CysSase	psgtsqcedgsagcpqdlevqpekgqlekgasgdkervwispdtpsrctwqlgrpmadsphyhtvptksp tkseqqadsrhnvidl	70 16 14
EcCysK	skifednslt	10
EcCysM	vstleqt	7
Rat-βSase ScStr4 Sp-CysSase EcCysK EcCysM	kilpdilrkIGNTPMVRINRIsknaglkcellakceffnaggSVKDRISLRMIEDAERAGTLKPG-dtII VGNTPLIALKKLpkalgikpqiyaklelynpggSIKDRIAKGNVETAERASGRIHPSrstif IGKTPLVYLNTVadgcvarvaaklegmepcsSVKDRIGFSNITDAERSGLITPGesvLf IGHTPLVRLNRIgngrilakvesrnpsfSVKCRIGANMIWDAERGVLKPG-veLV IGNTPLVKLORMgpdngsevwlklegnnpagSVKDRAALSMIVEAERRGEIKPG-dvLt	139 77 73 65 65
Rat- ßSase	$\verb"EPTSCHTGIGLALAAAVKGYRCIIV#PEKMSMEKVDVLRALGAEIVRTptnarfdspeshvgvawrlkne"$	209
ScStr4	EPTSCHTGIGLALIGAIKGYRTIITLDEKMSNEKVSVLKAIGAE IIRTpt a aawdspeshigvakklek e	147
Sp-CysSase	EPTSGNTGIGLAFIAAAKGYKLIITMPASMSLERRTILKAFGAKLILTdpakgmkgav qkaeeirdkt	141
EcCysk BoCush	EXISTINITALAIVARARGIRLIILIREFINSIERRALLARARAIVLITEGARGMEGAIGARGAIGARGAIVASADE	135
ACCYSR		100
	•••••	
Rat- β Sase	ipnshILDQYRNASNPLAEYDDTAEEIlqqcdgkvdmlVASAGTGGTITGIAEKIKEkcpgckii	274
ScStr4	ipgaviLDQINNMMNPEARYFGTGREIqrqledinifdniravVAGAGTGGTISGISKYLKEqndkiqiv	217
Sp-CysSase	-pnsyllogremping the war to the second secon	204
RCCYBR	kILDOFNNPDNPYAHYTTTGPHTwggtggrithfVSSNGTTGFTTGV8HFMRHgskovt1-	195
Lecybr		200
_	• • • •	
Rat-βSase	gvdpegsilaepeelnqteqtayevegigydFIPTVLDRAVVDRWFKSNDDDSFAFARMLISQEGLL	341
ScStr4	gaapigsliaqpeninktaitdykvegigydrvpevLDkkLiDvwikTDDkFBFKYARCLINNEGVL	284
Sp-CysSase	svaveptdspyjagalageeikogphriggigagervalleriteriteriteriteriteriteriteriteriterit	268
EcCysk	VglqpeegssipgirrwpteYLPGIFNASLVDEVLDIHORDAENTMRELAVREGIF	251
Dat Baasa		
Ract-poase	Consequence was a general tender to the second	408
SD-CV8Sase	VGISSGAAAAAIKvakrpenagklivAVFPSFGERVISsvlfdsvrkeaesmvies	324
EcCvsK	aGISSGAAVAAALKlgedesftnkniVVILPSSGERVLStalfadlftekelgg	322
EcCysM	cGVSSGGAVAGALRvakanpdavvVAIICDRGDRYLStgvfgeehfsqgagi	303
_		
0-		
Rat-pSase	vqeisisapitviptvtcentialirekgidqapvvnesgailgmvtlgnmlsslla	465
ScStr4	seenyaaviynatvnaininpvvsvnetanvtavininkangiaqipviteagkisgivtiselliklsi	424
Pat-Beaco		
202+~1	gkvipsdevckvlykqikpinitdtigmishilemdhfalvvheqiqsrdqawsgvvggptdrnngvssk	535
000LI¶	monn anerkykytaikkinninävssynenksykkiikidenskisäinrileknssävitägikpi	493
Rat-β Sase ScStr 4	qlmvfgvvtaidllnfvaareqtrk hivtkmdllsyla	560 506

FIG. 5. Sequence alignments of cystathionine β -synthases and cysteine synthases. Rat- β Sase, rat cystathionine β -synthase (33); ScStr4, cystathionine β -synthase encoded by the *S. cerevisiae STR4* gene; Sp-CysSase, spinach cysteine synthase (28); EcCysK, cysteine synthase encoded by the *E. coli cysK* gene (4, 19); EcCysM, cysteine synthase encoded by the *E. coli cysM* gene (32). The alignment was optimized by using the program of Schuler et al. (29). Blocks of similarities are in uppercase letters and boxed. Residues shared by at least three proteins are in bold letters. Black circles indicate residues strictly conserved in the five sequences. Open circles indicate the conserved residues of cystathionine β -synthases in the C-terminal extension. Possible pyridoxal binding sites are marked by arrows.

tor, pyridoxal phosphate, which forms a Schiff base with the ϵ -NH₂ moiety of a lysine residue. The lysine residues of rat and *E. coli* cystathionine γ -lyases identified as being bound to pyridoxal phosphate (22) are aligned with a lysine residue in the other proteins. This suggests that the pyridoxal phosphate cofactor of the cystathionine γ -lyase and homocysteine synthase from *S. cerevisiae* could be bound to the lysine residues indicated by an arrow in Fig. 6. By contrast, the pyridoxal phosphate binding site was identified neither for the cysteine synthases nor for the cystathionine β -synthases. However, the alignment depicted in Fig. 5 reveals

that only two lysine residues were strictly conserved between all of these enzymes (corresponding to lysines 41 and 52 of the yeast cystathionine β -synthase). Thus, given the similarity of these enzymes, it is possible that one of these two lysine residues is the pyridoxal phosphate binding residue.

DISCUSSION

The yeast cystathionine β -synthase- and cystathionine γ -lyase-encoding genes were cloned by functional comple-

Rat-YLyase ScStr1 EcMetC EcMetB ScMet25	ccgaahlYanSCHPTENCTARAV tlqesdkfatkaihagehvdvhgsviepislsttfkqsepanaigtyeYanSCHPTENCTARAV adkkldtqlvnagrskkytlgavnsviqrasslvfdsveakkhatrnrangelfYGENGTITEFSLOOM trkqatiavrsglnddeqygcvvppihlsstynftgfneprahdYanCHPTENVURAL pshfdtvqlhagqenpgdnahrsravpiyattsyvfenskhgsqlfglevpgyvTSAFQHPTSNVLESRI	40 64 70 60 70
Rat-YLyase	ALDOAkhcltfarglaatttithllka-GDEVICMDEVYGOTnryfrrvasefglkisfvdcsktklle	109
SCSTI	anima and a second se	140
ReMetC	With a south of the said but with the south of the south of the state of the south	130
ScMet25	ARLEGGaaalavssgqaaqtlaiqglahtGDNIVSTSYLYGGTynqfkisfkrfgiearfvegdnpeefe	140
	•••	4
Rat- <i>γ</i> Lyase	aaitpq TLIVWIETPINPTLKIADI kacaqivh kh kdiiJ VVDNIFmsa-y iqr DLA K ADI CMCKA	175
ScStr1		201
EcMetC		105
ScMet25	kvfderTKAVYLETICNPKYNVPDFekivaiahkhgipVVVDNTFgaggyfcqPIKYGADIVTHBA	206
	•• •• •	
Rat- <i>Y</i> Lyase	TRTMCHEDVVNGlvsvnsddlnerlrflqns	212
ScStr1	TETIMGHEDVVIGvlatnnkplyerlqflqnaIGAIP	238
EcMetC	TXYIVGHSDAMIGtavcnarcweqlrenayl	243
EcMet B	TRYIMGHEDVYAGVViakdpdvvtelawwannIGVTG	232
ScMet25	TENTIGE CTTIGE ivdsgkfpwkdypekfpqfsqpaegyhgtiyneaygnlayivhvrtellrd ig EE	276
	• • • • • • • • • • • • • • • • • • • •	
Rat- <i>Y</i> Lyase	SPFDCYICCRGLKHCrsgwrntfqdgmavarflesnpr-VEKVIYPGIPSHPCHElakrsaracpgmvsf	281
ScStr1	${\tt SPFDAWLTHRCLKTL}hlrvrqaals {\tt ankiaeflaadken v X av N r POLKTHPN Y D v v l k qhrdal {\tt g} g m i$	308
EcMetC	DADTATITSECERTLgvrlrqhhesslkvaewlaehpq-VARVNHPALPGSKGHEfwkrdftgssglfsf	312
EcMetB	GATDSYLLINGLETLvprmelaqrnaqaivkylqtqpl-vKKLYHPSLPENCOHFiaarqqkgfgamlsf	301
ScMet25	NPFASFLLLQGVETLslraerhgenalklakwleqspy-VSNVSTFCLASHSHHEnakkylsngfggvls	345
		240
Rat-JLY888	y-ikgLiqnaqviiknikiiaiaesiggyesiaelpalminasvpekdratigisdt	340
SCSTI	sirirggaeaasriasstriitiaesiggiesitevpavminggipkearegsgvidd	271
KCMGTC News	vikkkiinteelanyionisiismayswggyesiitanqpentaaripdgelusgi	3/1
ECMOLS Severas	e	415
SCRUT23	IYVKUIPHAUKECUPIKIBYAYVVHIKIASHIAHVYUAKEIVIAPYICUHKYIHUKEKIASYVLKUMAK	413
Rat- <i>Y</i> Lyase	• • • ESVGEDEKDELEDEggalkaahp	364
ScStr1	ISVGIEDTDDLLEDIkgalkgatt	393
EcMetC	LEIGLEDVDDLIADLdagfariv	394
EcMet B	ISTCIEDGEDLIADLengfraankg	385
ScMet25	VSVGIEFIDDIIADFggsfetvfaggkp	443

FIG. 6. Sequence alignments of cystathionine γ -lyases, cystathionine γ -synthase, cystathionine β -lyase, and homocysteine synthase. Rat- γ Lyase, rat cystathionine γ -lyase (9); ScStr1, cystathionine γ -lyase encoded by the *STR1* (*CYS3*) gene (24); EcMetC, cystathionine β -lyase encoded by the *metC* gene from *E. coli* (2); EcMetB, cystathionine γ -synthase encoded by the *metB* gene from *E. coli* (2); ScMet25, homocysteine synthase encoded by the *MET25* gene from *S. cerevisiae* (16). The alignment was optimized by using the program of Schuler et al. (29). Blocks of similarities are in uppercase letters and boxed. Residues shared by at least three proteins are in bold letters. Black circles indicate residues strictly conserved in the five sequences. The putative pyridoxal binding site is shown by an arrow.

mentation of strains bearing a mutated allele at the STR1 or STR4 gene. Disruptions of each of these genes gave viable haploid strains exhibiting a cysteine requirement. This auxotrophic requirement cannot be relieved by supplementation with other organic sulfur compounds, such as homocysteine and methionine. These results confirm our genetic analysis of sulfur metabolism in yeast cells (5): in S. cerevisiae, the sulfur atom resulting from the reduction of sulfate into sulfide is incorporated only in a four-carbon chain yielding homocysteine. Homocysteine is then used as a precursor for both cysteine and methionine synthesis. Sulfur metabolism in S. cerevisiae thus appears to be quite different from that existing in enterobacteria, in which the synthesis of cysteine is the first committed step of organic sulfur metabolism. Systematic sequence comparison of enzymes catalyzing the transsulfuration steps, including those allowing the incorporation of inorganic sulfur into a carbon chain, revealed the existence of two separate groups. The first one contains cystathionine β -synthases and cysteine synthases, while the second one contains homocysteine synthase, cystathionine γ -synthase, cystathionine β -lyase, and cystathionine γ lyases. This partition of transsulfuration enzymes, based on exclusive sets of sequence motifs, appears to be mainly correlated at the functional level with the length of the carbon chain of the amino acid substrate. Indeed, the first family is exclusively composed of enzymes catalyzing the β -replacement reactions of three-carbon-chain amino acids. The second family appears to be more heterogeneous. It is composed of enzymes catalyzing γ -replacement reactions of four-carbon-chain amino acids and the γ cleavage of cystathionine. However, this family comprises also the enzyme catalyzing the β cleavage of cystathionine. The presence of cystathionine β -lyase in this second family could reflect the history of the emergence of this enzyme (see below). The strong similarity displayed by the members of each family suggests that all transsulfuration enzymes may have evolved from only two ancestral proteins which could have been pyridoxal phosphate enzymes. Considering the alignment of cysteine synthases and cystathionine β -synthases, it seems reasonable to speculate that the latter could have been achieved by the fusion of an additional C-terminal domain to a primitive cysteine synthase. This specialization could have been favored by substrate ambiguity of such a primitive cysteine synthase. Accordingly, it must be stressed that substrate ambiguity remains in modern cysteine synthases. For example, the E. coli cysM-encoded product was shown to be capable of catalyzing both cysteine and S-sulfocysteine synthesis (17). The data presented are also consistent with a model in which the common ancestor of the second group of transsulfuration enzymes could have been a primitive homocysteine synthase. Traces of the broad reactivity of such an ancestor homocysteine synthase emerge in contemporary metabolism. The modern homocysteine synthase is indeed capable of catalyzing in vitro different reactions, such as sulfhydrylation of O-acetylserine (37). In vivo studies have also demonstrated the capacity of the yeast homocysteine synthase to catalyze the conversion of ethionine into methionine (6).

According to these speculative statements, direct incorporation of sulfide into both a three- and a four-carbon chain must have coexisted in primitive organisms. The building up of the transsulfuration pathway(s) bridging the biosyntheses of cysteine and homocysteine could have been subsequently achieved by the enzymatic recruitment of cysteine synthase and homocysteine synthase. As indicated by the overall similarities exhibited by the transsulfuration enzymes, such transitions to new catalytic functions could occur through only a few genetic changes. This evolution could have been retained especially to favor the coordinate control of sulfur amino acid biosynthesis. It is worth stressing that the hypothesis of the coexistence of ancient cysteine and homocysteine synthases is strongly reinforced by experimental data acquired on sulfur metabolism in enterobacteriaceae; for example, Simon and Hong (31) have discovered the latent capacity of E. coli to synthesize homocysteine from O-succinylhomoserine, bypassing the cystathionine pathway. This reaction was found to occur in a methionine prototroph revertant of a cystathionine γ -synthase-deficient strain. This new mutation defines a gene, metQ, encoding a 41-kDa polypeptide (23) that could be a homocysteine synthase.

Other constraints seem to have been imposed on the evolution of this family of proteins, perhaps to meet the starvation experienced by all microorganisms in natural environments. Indeed, yeast cystathionine γ -lyase is completely devoid of cysteine residues, and cystathionine β -synthase of the same organism contains only one cysteine residue. By contrast, the rat cystathionine γ -lyase and cystathionine β -synthase contain 14 and 13 cysteine residues, respectively. This observation could be related to the fact that in *S. cerevisiae*, the cysteine content strictly depends on the reactions catalyzed by these enzymes, whereas in mammals, cysteine is provided directly by the diet.

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