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Production and Characterization of Fully Selenomethionine-Labeled *Saccharomyces cerevisiae*

LAURENT OUERDANE AND ZOLTÁN MESTER*

Institute for National Measurement Standard, National Research Council Canada, Ottawa, Ontario, Canada K1A 0R6

This paper reports, for the first time, a quantitative replacement of methionine (Met) by selenomethionine (SeMet) at >98% substitution, with up to 4940 μg of SeMet/g of yeast obtained for the entire protein pool of a wild-type yeast grown on a SeMet-containing medium. The incorporation of selenium in yeast proteins, in the form of selenomethionine, and the influence of various organic and inorganic Se and S sources present in the media were monitored during the growth of a wild-type *Saccharomyces cerevisiae*, which allowed the optimization of the composition of a fully defined synthetic growth medium that ensured maximum SeMet incorporation. Quantitation of SeMet and Met was performed by species-specific isotope dilution GC-MS. The use of ascorbic acid and a minimum concentration of cysteine (5 μg/L) was found to be beneficial to achieve incorporation by limiting the oxidative stress due to the presence of selenium. Except for small amounts of cysteine, no other sources of sulfur were necessary to achieve yeast growth. In a medium containing Se(VI), the maximum replacement of Met with SeMet was 50%, which is considerably higher than that obtained with the current commercial Se yeast formulations. For yeast grown in a Met-free defined medium, which was supplemented with SeMet, nearly total replacement of Met with SeMet could be achieved. The fully Se-labeled yeast could be an important tool for the study of eukaryotic protein structures both by mass spectrometry and by X-ray crystallography through selenomethionine single- and multiple-wavelength anomalous dispersion (SAD and MAD) phasing. In addition, a particular yeast strain, BY4741, that cannot synthesize Met using inorganic sulfur (*met15*Δ0) was shown to produce SeMet in the presence of inorganic selenium. This might indicate that the incorporation of inorganic selenium salts [Se(VI) and Se(IV)] is obviously not occurring exclusively through the same biological pathways as for sulfur. The reduction of inorganic Se to hydrogen selenide (H_2Se), its reactions with organic compounds present in the yeast or in the media, and the possible metabolization through unspecific enzymatic pathways (such as transsulfuration) could also be of considerable importance in the production of selenoamino acids during yeast growth.

KEYWORDS: Selenium; selenomethionine; methionine; yeast; *Saccharomyces cerevisiae*; selenium incorporation; methionine auxotroph; selenide

INTRODUCTION

Because of its chemical similarity to sulfur, selenium can also be metabolized by living organisms. In mammals, it appears that the presence of this element is even essential for healthy development, as it is translationally incorporated in the form of an amino acid, selenocysteine, into selenoproteins that perform specific and essential functions in the organism (*1*−*4*). Selenium-containing compounds have also been reported to have anticarcinogenic properties (*5*−*9*). Because selenium availability in food grown in some regions can be very low, the use of Se supplements to improve animal and human nutritional intake is now widespread.

It has been observed that yeast can accumulate selenium in its proteins through replacement of methionine by selenomethionine (*10*−*12*). Indeed, inorganic selenium [Se(VI), Se(IV)] is converted into compounds containing Se in its reduced forms (organic selenium) that are much less toxic and more bioavailable than the original salts and, therefore, have an improved nutritional quality (*13*). The use of yeast for the production of Se-enriched supplements at an industrial scale is much more convenient than the use of other organisms such as plants for accumulating selenium. This has raised interest in developing production techniques that would allow maximum reduction and incorporation of inorganic selenium in yeast. However, the

* Author to whom correspondence should be addressed (e-mail Zoltan.Mester@nrc.ca).

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particular mechanisms underlying the metabolization and toxicity of selenium (and its similarities and differences with sulfur metabolic pathways) are still unclear, although a few recent works have raised some important issues about selenium fate in yeast (*14, 15*).

Developing highly selenium enriched yeast would be of great interest for Se metabolomic studies and a potential source of eukaryotic posttranslationally modified proteins incorporating SeMet instead of Met, which would allow protein structural analysis by X-ray crystallography (*15*−*17*).

Hyphenated techniques are usually employed to determine the nature and the extent of Se incorporation in yeast. Typically, they consist of chromatography interfaced with various elemental and molecular mass spectrometry approaches. These techniques have been used extensively to identify (*11, 18*−*28*) and quantify (*29*−*35*) selenocompounds in yeast.

The purpose of this study was to develop a method to incorporate maximum amounts of reduced selenium, especially SeMet, in yeast. To achieve this, all aspects of the yeast media compositions were optimized including all sources of sulfur and selenium. During the industrial processing of Se-enriched yeast, the objective is to incorporate in yeast the maximum amount of inorganic Se (inexpensive source of selenium) and transform it into an organic Se form (SeMet being the preferred chemical form). However, industrial processing can barely achieve 30−45% replacement of Met by SeMet. Therefore, the aim of this study is to investigate whether complete incorporation of SeMet instead of Met in a wild strain of yeast is possible and also to obtain some clues on the incorporation pathway of inorganic Se, which in turn could give some indications on what kind of yeast strain could be used to lower Se toxicity and to increase SeMet incorporation in yeast during its industrial processing.

MATERIALS AND METHODS

A wild-type strain of baker's yeast, *Saccharomyces cerevisiae*, was isolated (selected after being grown in a Petri dish) and used in all of these experiments. All chemicals were of analytical grade and compatible with cell cultures.

As commercially prepared yeast growth media, such as YEPD, are usually based on fungus peptone or yeast extracts, they contain many sources of sulfur and possibly selenium. Therefore, synthetic defined media optimized for *S. cerevisiae* were prepared to control sulfur during yeast growth. All prepared media had the same chemical constituents except that sources of sulfur and selenium were varied.

Instrumentation. An ELAN DRC II ICPMS (PE-Sciex, Thornhill, ON, Canada) equipped with a Ryton spray chamber and cross-flow nebulizer was used for the detection of selenium and sulfur. Hydrogen was used as a collision gas, and ICPMS parameters, nebulizer gas flow, rf power, lens voltages, and hydrogen gas flow, were optimized daily to get the best S/N ratio for S and Se. A Hewlett-Packard HP 6890 GC (Agilent Technologies Canada Inc., Mississauga, ON, Canada) fitted with a DB-5MS column (Iso-Mass Scientific Inc., Calgary, AB, Canada) was used for the separation of the Met and SeMet in the derivatized yeast extracts. Detection was achieved with an HP model 5973 mass-selective detector (MS). A CEM (Matthews, NC) MDS-2100 microwave digester equipped with Teflon vessels was used for closed-vessel high-pressure decomposition of yeast for total Se and S determination.

Growth Media Preparation. Salts, nitrogen source, and carbohydrate source were mixed together in an appropriate volume of water to obtain the following final concentrations: 1000 mg/L of H_2KPO_4, 500 mg/L of $MgCl_2$, 100 mg/L of NaCl, 100 mg/L of $CaCl_2$, 5000 mg/L of NH_4Cl, and 20000 mg/L of dextrose. This solution was then autoclaved at 121 °C for 35 min and stored aseptically. After the mixture had cooled, previously prepared vitamins, trace elements, and amino acid solutions were added with syringes through 0.2 μm sterile filters to reach the final concentrations detailed in **Table 1**. The latter were added to the media through syringes and filters after the media were autoclaved to prevent their denaturation. This final solution is called the base medium and will be used in all of the experiments described in this study. Supplementary additions of compounds to the base media were also made through syringes and 0.2 μm sterile filters. All manipulations of glassware containing growth medium and/or yeast were performed inside a laminar flow hood, and the manipulating tools were sterilized with a flame or in the autoclave.

Table 1. Concentrations of Amino Acids, Vitamins, and Trace Elements Present in the Base Medium

vitamin	mg/L	trace element	mg/L	amino acid	mg/L
biotin	0.002	H_3BO_3	0.5	adenine	10
pantothenate, Ca	0.4	$CuCl_2$	0.04	L-arginine, HCl	50
folic acid	0.002	KI	0.1	L-aspartic acid	80
inositol	2	$FeCl_3$	0.2	L-histidine	20
niacin	0.4	$MnCl_2$	0.4	L-isoleucine	50
PABA	0.2	Na_2MoO_4	0.2	L-leucine	100
pyridoxine, HCl	0.4	$ZnCl_2$	0.4	L-lysine, HCl	50
riboflavin	0.2			L-phenylalanine	50
thiamin, HCl	0.4			L-threonine	100
				L-tryptophan	50
				L-tyrosine	50
				uracil	20
				L-valine	140

Table 2. Type and Volume of Media Replaced after Collection for Each Flask[a]

	day								
	1	2	3	4	5	8	10	12	16
replaced vol (mL)	50	50	50	50	50	90	90	90	100
expt A (S+)	S−	S−	S−	S−	S−	S−	S−	S−[b]	
expt B (S−)	S−	S−	S−	S−	S−	S−	S−	S−[b]	
expt C (S+)	S+	S+	S+	S+	S+	S−	S−	S−[b]	
expt D (S+)	S+	S+	S+	S+	S+	S−	S−	S−[b]	

[a] For each experiment, the medium used to initiate yeast growth is given in parentheses. [b] Medium without sulfate that is deprived of cysteine also.

Fermentation Experiments. *First Experimental Setup (A*−*D).* The first set of experiments was prepared using a semicontinuous fermentation process. Two different media were employed to prepare the four experiments, one containing the base medium with 50 mg/L of Na_2SO_4 added (S+) and the other one without Na_2SO_4 (S−). A solution of concentrated ascorbic acid was added to all of the media (S+ and S−) to reach a final concentration of 500 mg/L. Small amounts (10 mg/L) of cysteine, a sulfur-containing amino acid, were also added to both media, so S− medium is not technically free of sulfur. However, cysteine was not put in the last replacing medium added on the 12th day of the experiments, which therefore resulted in a 10-fold decrease of cysteine concentration in the flasks. Either solution S+ (experiments A, C, and D) or solution S− (experiment B) was poured into four sterile flasks (250 mL) corresponding to each experiment (A, B, C, or D), and each flask was inoculated with a similar amount of yeast. The flasks were stoppered with sterile cotton balls, and they were put into an incubator (150 rpm and 22 °C) to grow for 3 days. The system was devised to keep yeast growing while being able to modify the nutrient concentrations in each flask and collect samples. As described in **Table 2**, parts of the yeast-containing media were collected, and the flasks were refilled with identical volumes on days 1−5, 8, 10, 12, and 16 (day 1 corresponds to the first yeast collection and medium replacement and, at day 16, yeast growth was stopped after the last collection). Each time, different volumes of selenomethionine or Na_2SeO_4 in solution in deionized water (with concentrations of 1000 and 4000 ppm, respectively) were added to the flasks through the replacement media to reach concentrations in media as described in **Figure 1**. For all of the experiments in this study, Se(VI) was chosen over Se(IV) as a source of inorganic selenium because, although it is more toxic, it is also more easily converted to organic selenium by the yeast than Se(IV) (*36*).

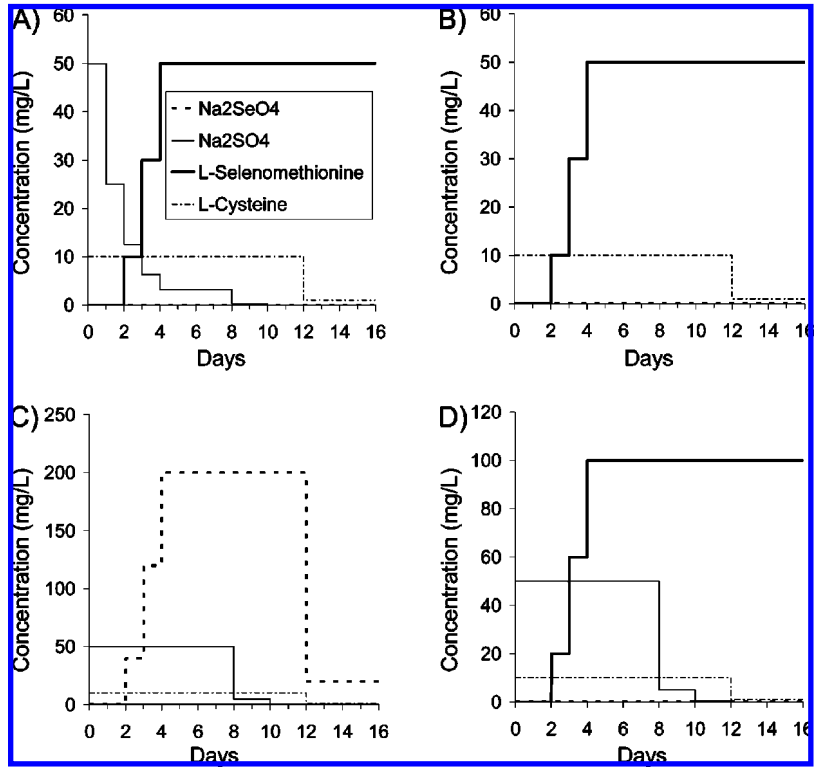

Figure 1. Expected variations of concentrations of Na_2SeO_4, Na_2SO_4, and SeMet in the feeding media of flasks 1 (**A**), 2 (**B**), 3 (**C**), and 4 (**D**) during yeast growth.

Second Experimental Setup (E, F). By taking into account the information obtained in these first experiments, another set of experiments was prepared to grow yeast that would contain the highest molar ratio possible of selenomethionine to methionine in proteins. These experiments were performed using either selenomethionine (experiment E) or Na_2SeO_4 (experiment F) as a selenium source. The optimized growth media consisted of the base medium described above with 5 mg/L cysteine to which were added selenomethionine (100 mg/L) and ascorbic acid (500 mg/L) in experiment E or Na_2SeO_4 (75 mg/L) in experiment F. During these new experiments, the medium compositions were not modified. After 1 or 2 weeks, the growth ended, and the yeast was collected. Ascorbic acid was found to decrease the toxicity of selenomethionine, probably because it reduces the amount of SeMet degradation products present and the redox perturbations due to selenol-containing compounds produced during SeMet metabolization in the yeast. Ascorbic acid also decreases the toxicity of inorganic selenium salt because it reduces a significant part of the Se(VI) [or Se(IV)] present in the media into elemental selenium, which is less toxic for the yeast. Consequently, ascorbic acid was not introduced in the medium of experiment F to ensure maximum availability of sodium selenate for the yeast.

Third Experimental Setup (G, H). The third set of experiments was prepared to compare the selenium incorporation between two specific yeast strains, BY4741 (MATa *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0) and BY4742 (MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0). BY4741 yeast is not able to use inorganic sulfur sources to grow as the gene *met15* (also known as *met25*) is depleted. These experiments were performed using either selenomethionine (experiment G) or Na_2SeO_4 (experiment H) as a selenium source. The optimized growing media were constituted of the base medium with a small amount of cysteine (10 mg/L) and methionine (20 mg/L) to which were added after the first day of growth selenomethionine (30 mg/L) and ascorbic acid (500 mg/L) in experiment G or Na_2SeO_4 (20 mg/L) in experiment H. After this addition, the medium compositions were kept identical. After the growth ended, about 5 days later, the yeast was collected.

Collection and Conditioning of Yeast Samples. When the yeast was collected, the flasks were shaken to suspend the yeast and the same specified volume for each flask was transferred to centrifuge tubes. The last day of all sets of experiments the total volume of medium solution was collected. The samples taken from the fermentation systems were centrifuged three times at 3000*g* for 5 min. Each time, the supernatant was removed and replaced by deionized water that was homogenized with the yeast before the next centrifugation. The end product, cleaned yeast, was transferred into vials and immediately frozen. The samples were lyophilized, and the dry weights of yeast were measured.

Total Selenium and Sulfur Determination. Four 5 mg subsamples of each freeze-dried yeast were accurately weighed into individual precleaned Teflon digestion vessels. After 0.9 mL of nitric acid and 0.1 mL of H_2O_2 were added, the vessels were capped and digested in a CEM MDS-2100 microwave oven. The heating conditions were as described by Yang et al. (*31*). After cooling, 0.25 mL volumes of the digested solutions were transferred to precleaned polyethylene screw-cap bottles and diluted to 10 mL with 1% HNO_3. Sulfur and selenium were quantified using ICPMS by standard additions of inorganic sulfur and selenium solutions (intensities were normalized using rhodium standard).

Met and SeMet Determination. The yeast extraction procedure used in this study followed that described by Wrobel et al. (*33*) with small modifications. In brief, 20 mg of yeast was spiked with 200 μL of 19.02 μg/g ^{13}C-enriched SeMet and 200 μL of 61.89 μg/g ^{13}C-enriched Met. After the addition of 17.6 mL of deionized water and 6 mL of methanesulfonic acid (final concentration of 4 M methanesulfonic acid), the contents were refluxed on a hot plate for 16 h. The derivatization procedure followed that reported by Haberhauer-Troyer et al. (*37*) and by Yang et al. (*31*). After derivatization and solvent extraction, the digested yeast was analyzed by GC-MS, and the amounts of selenomethionine and methionine per gram of yeast were determined by a species-specific isotope dilution calculation as already described (*31*).

RESULTS AND DISCUSSION

Semicontinuous Fermentation Experiments. During the semicontinuous fermentation experiments, four different dynamic approaches were used to obtain information on the best way to incorporate the maximum amount of selenomethionine in yeast proteins. These experiments were designed to study

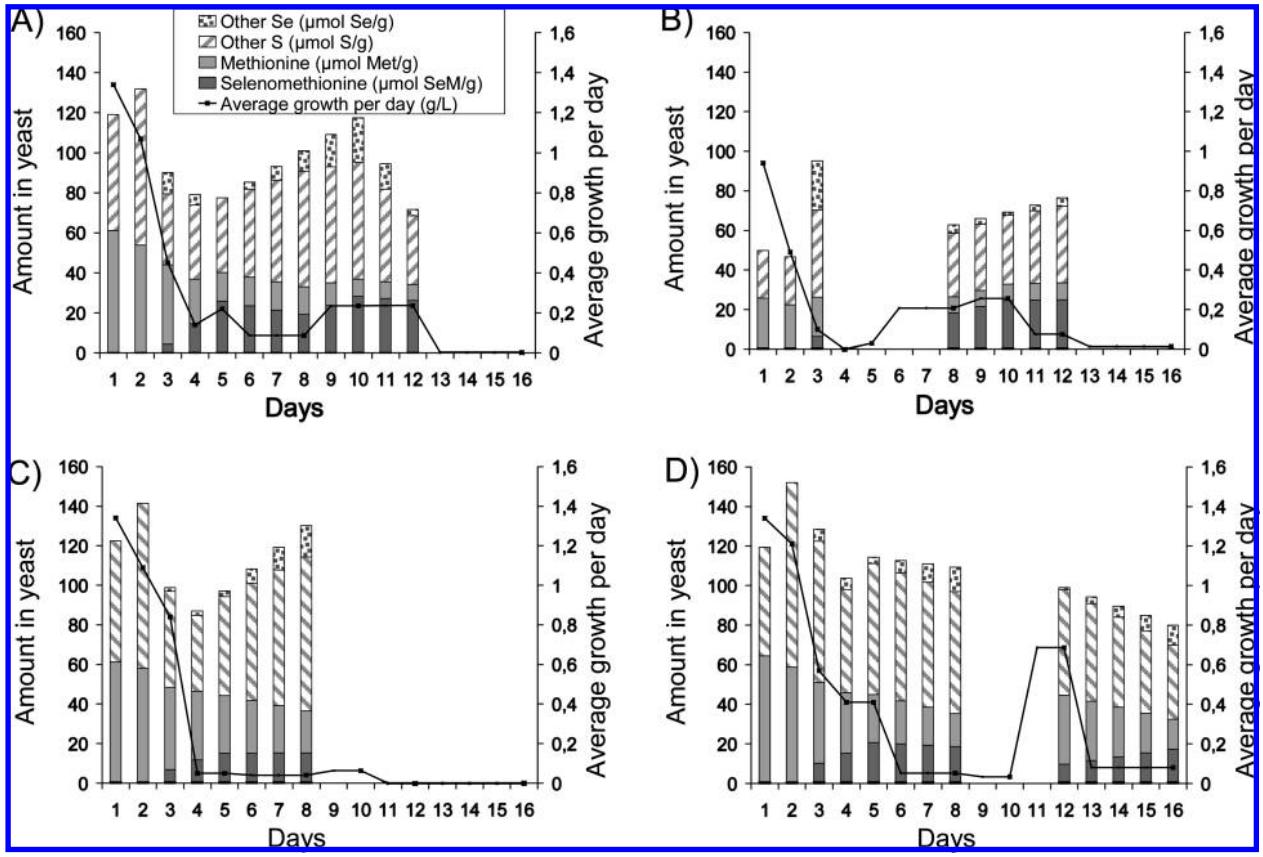

Figure 2. Measured growth rates and amounts of methionine, selenomethionine, other forms of sulfur, and other forms of selenium per gram of yeast at different times during their growth. Missing data are due to a low amount of yeast material on that day because of a lack of growth. Values for days between collections are extrapolated. The relative standard deviations on these amounts ($n = 3$) were 5−10% during the steady state period (days 1 and 2 and from day 9 to day 16) and 10−15% during the transition state (from day 3 to day 8).

the impact of decreasing sulfur and increasing selenium concentrations on the metabolism of these elements in yeast.

The concentrations of sulfur and selenium were chosen on the basis of preliminary toxicity trials to obtain sufficient biomass production.

In experiments A, C, and D, as described under Materials Methods, the flasks were initially filled with S+ media (before day 1) so the yeast started to grow in the presence of sulfur (Na_2SO_4), whereas, in experiment B, the flask contained no sulfate (**Table 2**). As a consequence, the total amount of sulfur and Met in the yeast of experiment B (**Figure 2B**) is 2−3 times lower than at the beginning of the experiments A, C, and D (**Figure 2A**,**C**,**D**). It is interesting to note that the absence of sulfate caused only a 40% decrease in growth rate. For these four experiments, the measured amounts of Met, SeMet, other forms of S, and other forms of Se present in the yeast when they were collected, as well as their growth rate, are all summarized in **Figure 2**.

At day 3, an important increase in the amounts of both S and Se in the yeast originally depleted in sulfur can be observed. The cumulative effects of sulfur depletion and selenium toxicity on sulfur availability for the yeast probably boost the mechanisms that are involved in sulfate assimilation (membrane transporters, etc.), which leads to faster Se incorporation than in the other yeast that day but stops also the growth on day 4 because of the toxicity of this newly absorbed selenium. Between days 5 and 8, the yeast originally depleted in sulfur started to grow again, which could be a sign of partial adaptation of the yeast metabolism to these Se-enriched and S-depleted growth media. It can be observed that the less sulfur the yeast media contained originally, the faster the yeast was affected by selenium toxicity but the faster, also, it would recover, that is, grow again. It seems clear that the yeast containing sufficient sulfur reserves at first just slows its metabolism (and so its growth) when toxic compounds (such as selenium species) are present in the media. The yeast probably recycles internal sources of accumulated sulfur before being affected, which explains this delay in the toxicity response. Compared to the yeast of experiment A (**Figure 2A**), the one in experiment B (**Figure 2B**) reached a steady state for the amount ratios of total Se/S and SeMet/Met more quicly. In the presence of more abundant sulfur sources, the yeast growing in a medium containing selenate (**Figure 2C**) or a high level of selenomethionine (**Figure 2D**) also showed a slower adaptation to selenium presence and a lower metabolization of this element.

The competition between selenomethionine and methionine (produced by the yeast using the sulfur sources in the media) during protein synthesis could explain the partial incorporation of SeMet in yeast. In the presence of selenium, yeast growth decreased by 5- (**Figure 2A**,**B**) to 20-fold (**Figure 2C**) compared to the original growth, but the amount of yeast collected was sufficient for analysis most of the time. After day 12, all of the yeast media were depleted in cysteine ($^1/_{10}$ of its original concentration), which resulted in the end of yeast growth in experiments A−C and in a severe reduction of growth in experiment D. Cysteine seems to be essential to yeast growth, and only a limited concentration of Se analogues of cysteine has been detected (*19, 38*) This is probably due to the toxicity of selenocysteine competing with cysteine metabolism and the fact that this selenoamino acid can easily degrade in highly toxic

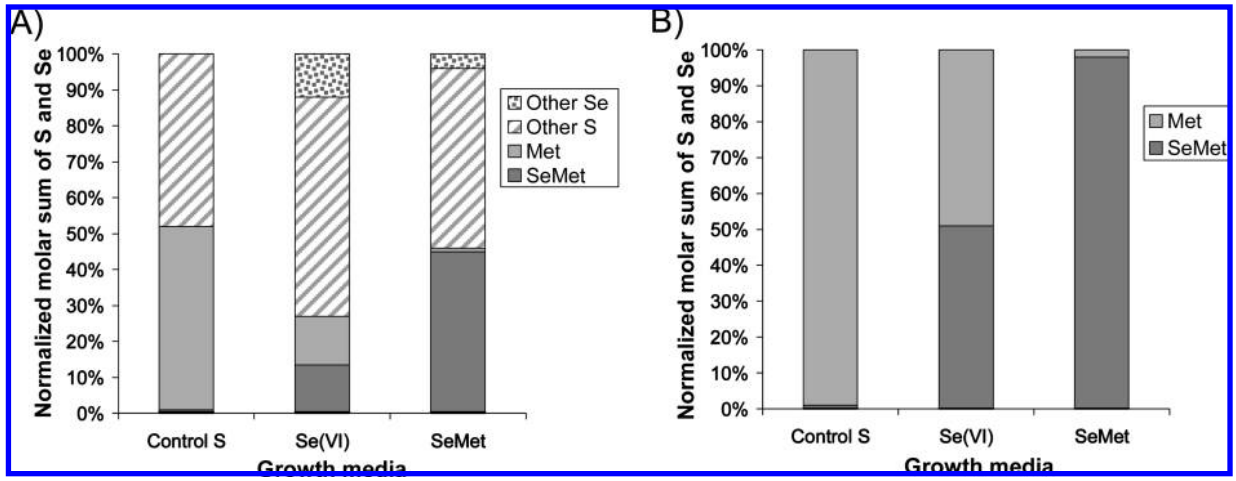

Figure 3. Maximum incorporations of total selenium (**A**) and SeMet (**B**) obtained for yeast grown in the presence of Se(VI) and SeMet compared to a control yeast grown with sulfur. The relative standard deviations of the raw amounts were 5−10% ($n = 3$) (**A**), whereas the relative standard deviations on the ratios were lower due to a better consistency of the ratios and were, respectively, 0.5, 4, and 1.5% for the control, Se(VI), and SeMet growth media.

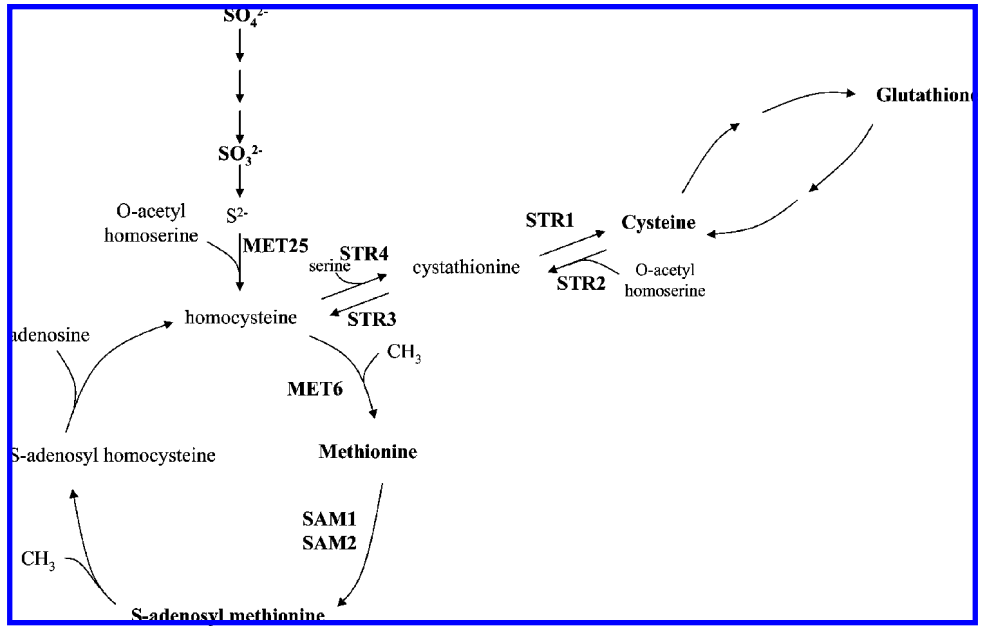

Figure 4. Simplified scheme of sulfur amino acid biosynthesis in *Saccharomyces cerevisiae* (*46*). Each arrow corresponds to a transformation driven by a particular enzyme.

hydrogen selenide (*14*). Once the medium contains a high level of selenomethionine, the metabolism of cysteine into a significant amount of methionine is unlikely as the yeast methionine pool is saturated (by selenomethionine). Therefore, the presence of cysteine in the growth media does not affect the selenomethionine to methionine ratio significantly. Similarly, the production of selenocysteine is unlikely because of the presence of cysteine. Consequently, a continuous presence of cysteine in the media of yeast growing to accumulate selenomethionine is recommended to prevent the biosynthesis and protein integration of selenocysteine and its degradation to toxic side products.

The day following selenomethionine addition into the media, the yeast is apparently metabolizing it into another Se species as selenomethionine does not account for all of the selenium present in the yeast. In experiment B, on day 3, only one-fourth of the selenium present in the yeast is present in the form of selenomethionine. Apparently, this selenium pool is actively participating in selenium toxicity as the following days the yeast grew only when it was finally able to keep selenium in the form of selenomethionine. It is conceivable that the toxic Se molecules produced in the early stage of yeast growth are actually Se-adenosyl selenomethionine and, especially, its side products (Se-adenosyl selenohomocysteine, etc.), as suggested by Malkowski and al (*15*). After a time of adaptation, the yeast seems to reduce the production of these metabolites, which coincides with the reduction of Se toxicity.

It is usually observed that most of the selenium in yeast is present in the form of selenomethionine in yeast proteins (*10, 11*). During experiments A−F, the yeast samples were also analyzed for free and protein-bound selenomethionine. This is a significant question as in some of the experiments SeMet was used in the growth media and, therefore, it is conceivable that SeMet would simply be accumulating in the cytosol without actually being used in the protein synthesis. Met/SeMet detected in the sample extracts obtained through sonication without acid hydrolysis corresponds to the "free" species, whereas the acid digestion provides information on the Met and SeMet built into the yeast proteins. Results obtained indicate that free selenomethionine

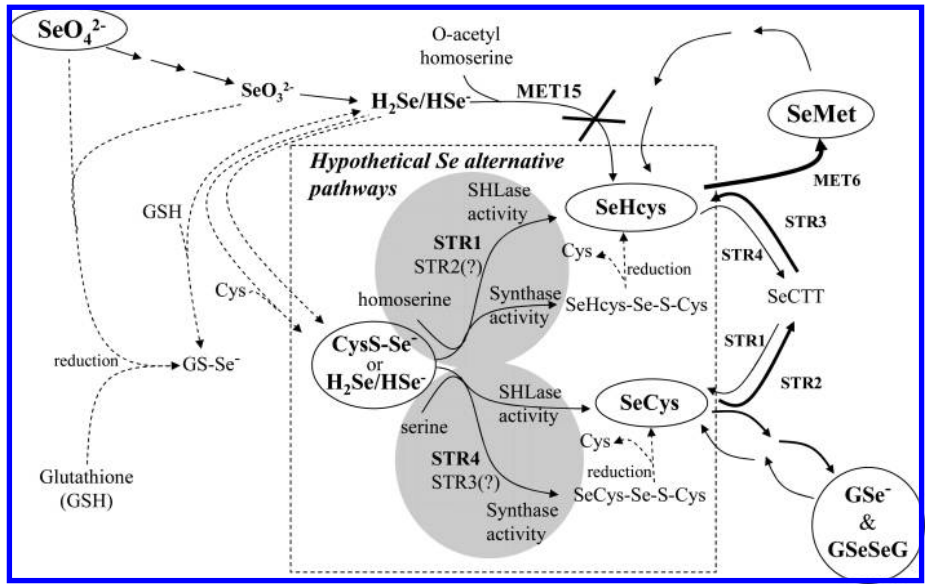

Figure 5. Possible mechanisms of formation of organic selenium amino acids in yeast strain BY4741 (*met15*Δ0). GSe^- and GSeSeG are, respectively, selenoglutathione and selenodiglutathione. Each solid arrow in this figure corresponds to a transformation driven by a particular enzyme, and each dotted arrow corresponds to a chemical reaction.

and methionine were <1% of their total concentrations in all of the experimental conditions. SeMet represents up to 80% of the Met/SeMet pool in experiments A and B (yeast grown on SeMet, **Figure 2A**,**B**), whereas the yeast is still growing, showwin that the incorporation of this selenoamino acid in yeast proteins is hardly contributing to selenium toxicity.

In experiment C (**Figure 2C**), yeast growth is very limited after the introduction of Na_2SeO_4, and the incorporation is less important than with SeMet. As expected, inorganic selenium salts are more toxic to yeast than SeMet [lower concentrations of Se(VI) were also tested and gave similar growth results and lower Se incorporation (data not shown)]. The presence of ascorbic acid could have also been a problem as it is involved in Se(VI) reduction to elemental Se(0) (red deposit seen in the flask), which could interfere with yeast growth.

In **Figure 2D**, it is interesting to note that, between days 8 and 12 in experiment D, the amount of selenomethionine (and total selenium) in the yeast decreased by a factor 3 and the amount of methionine increased by a factor 2.5; at the same time, the yeast medium was being depleted in sulfur (from 50 to 0 mg/L Na_2SO_4) and the level of selenomethionine in the medium kept high (100 mg/L). This phenomenon is probably due to modifications in yeast metabolic pathways that, as a response to the selenium toxicity, would reduce or stop the absorption of SeMet (exogenous source) and would metabolize the sulfur present in other forms in the yeast, cysteine or other accumulated forms of sulfur, to produce methionine. This is not observed in experiments A and B because, in those cases, Na_2SO_4 concentration is decreasing quickly or is already zero and, therefore, the yeast seems not to contain enough sulfur to initiate this conversion.

From a more general point of view, once selenium is added to yeast media (day 3 and after), it has been observed that the molar sum of S and Se in the yeast is always between 60 and 125 μmol/g of yeast whatever the conditions are. This shows that these elements are highly regulated and that the concentrations of the main compounds, Met/SeMet and cysteine, have to be within a certain range for the yeast to be viable. Also, in the presence of selenium, the fraction of other sources of S (mainly cysteine-containing compounds) is usually increasing and the level of methionine is decreasing, which proves once again that Met presence is not essential, whereas cysteine is essential not only for yeast growth but also for yeast detoxification. Finally, it can be observed that the molar sum of SeMet and Met never drops dramatically and, therefore, the presence of either one of these two amino acids is still essential for yeast growth and protein production.

Growth of Fully SeMet-Labeled Yeast. Using the observations from experiments A−D, batch growth experiments were set up aiming for maximum SeMet incorporation. The conditions for experiments E and F are described under Materials and Methods. In experiment E, the yeast contained >98% SeMet versus Met (**Figure 3**), with amounts of 4940 μg of SeMet/g of yeast. According to the literature, this level of incorporation has never been observed in wild-type yeast (*S. cerevisiae*) and just approached once in genetically modified yeasts (*15*). Fully SeMet-labeled yeast could be important for the study of posttranslationally modified eukaryotic proteins using X-ray crystallography. In experiment F, the yeast analyzed contained a little more than 50% SeMet (versus Met) (**Figure 3**), which is the highest percentage ever reported for a yeast grown on inorganic selenium.

In the experiments when the yeast had grown in the presence of selenate, deposits of red particles could be observed in the media as well as in the yeast. These particles were collected with the yeast. This deposit is very likely to be Se(0) from the reduction of selenate in the yeast. Particles of elemental selenium have been found to have efficiency similar to that of SeMet in up-regulating selenoenzymes for mice feeding (*39*), which makes this form of selenium an interesting complement to SeMet-enriched supplements.

Maximum selenium incorporations obtained for the yeast are summarized in **Figure 3**. Except the ratios between SeMet and Met, the relative amounts of sulfur and selenium are similar to the last days of yeast growth in the semicontinuous fermentation experiments (A−D) and, therefore, lead to the same observations.

The method developed in this paper allowed incorporation of high levels of selenium in yeast. The use of sophisticated analytical approaches such as isotope dilution MS made it possible throughout yeast growth to follow the incorporation of selenium, in the form of SeMet, in yeast proteins, which in turn allowed the optimization of growth media composition.

For the first time, a complete replacement of Met by SeMet was obtained in all of the proteins of a wild-type yeast. Apparently, yeast can grow with proteins containing only SeMet instead of Met, as long as the toxicity of potential SeMet metabolites is limited by the use of appropriate growing conditions.

Incorporation of Selenium in a Yeast Strain Incapable of Assimilating Inorganic Sulfur. Selenium incorporation experiments were performed on yeast strains BY4741 and BY4742. As described uner Materials and Methods, both yeasts were grown with SeMet or Na_2SeO_4. Yeast BY4741 is unable to transform inorganic sulfur into organic forms (e.g., Met, Cys), which makes it impossible to grow without the presence of Met or another source of organic sulfur. To illustrate this, a part of the sulfur pathway in *S. cerevisiae* is described in **Figure 4**. Astonishingly, 16% of SeMet (790 μg of SeMet/g of yeast) was observed in the SeMet/Met pool of yeast strain BY4741 that was grown on Na_2SeO_4. Even though it is less than the amount observed in yeast BY4742 (23%), this presence of SeMet cannot be explained by "normal" sulfur pathway of selenium, as described in **Figure 4**. Therefore, alternative pathways are most likely open for selenium including, perhaps, some Se-specific pathways. According to former studies (*14, 40*−*43*), hypothetical pathways that could justify the presence of SeMet in the yeast BY4741 grown with selenate have been summarized in **Figure 5**.

It is believed that the formation of selenoamino acids from inorganic selenium sources follows the same pathway as sulfur. Inorganic sulfur, enzymatically reduced to H_2S, reacts with *O*-acetylhomoserine catalyzed by MET15 encoded enzyme to form homocysteine (**Figure 4**). However, the yeast that cannot express the MET15 gene can still produce selenoamino acids with inorganic selenium, whereas it cannot produce sulfur amino acids with inorganic sulfur. Therefore, one can hypothesize some other enzymes could use HSe^- or $RSSe^-$ as nonspecific substrates. Earlier studies of the transsulfuration pathway of *S. cerevisiae* (*40, 42, 43*) suggested that many of the enzymes such as ones encoded by STR genes involved in this pathway retained evolutionary vestigial catalytic activities, such as sulfhydrylase activity (**Figure 5**) and could use various types of substrates in vitro. However, these peripheral reactions could not be observed in vivo as the *met15* mutated yeast strain is unable to produce sulfur amino acids. Therefore, these reactions are not considered to be likely sulfur pathways in *S. cerevisiae*. However, the much higher solubility and reactivity of H_2Se ($pK_{a1} \sim 3.8$) compared to H_2S ($pK_{a1} \sim 7.0$) at yeast physiological pH (5 to 6.5) could support the hypothesis that H_2Se could be a potential substrate of the residual sulfhydrylase activity of these enzymes. This creates a bypass route for inorganic Se incorporation into amino acids that does not involve MET15. Therefore, substrates such as H_2Se, RSSeH, *S*-cysteinyl selenide anion ($CysS-Se^-$), or *S*-glutathionyl selenide anion ($GS-Se^-$) could react, forming selenocysteine (SeCys) or selenohomocysteine (SeHcys) in a single step (**Figure 5**). Many of the metabolites of this pathway have already been observed in *S. cerevisiae* grown with inorganic selenium including SeCys and SeHcys residues (*44*) and volatile Se-alkyl species (*45*).

In this study we confirmed that global replacement of Met with SeMet is possible and that the yeast is still viable. We also found that the incorporation of inorganic selenium could occur through pathways different from sulfur, potentially, through nonspecific enzymatic residual catalytic activities which might utilize selenides. This point will have to be studied further to pinpoint the key enzymes involved.

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