Whole-Cell Detection by ^{13}C NMR of Metabolic Flux through the C_1-Tetrahydrofolate Synthase/Serine Hydroxymethyltransferase Enzyme System and Effect of Antifolate Exposure in *Saccharomyces cerevisiae*[†]

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Received February 8, 1994; Revised Manuscript Received April 11, 1994®

ABSTRACT: Folate-mediated one-carbon metabolism is critical for the synthesis of numerous cellular constituents required for cell growth. A potential source of one-carbon units is formate. This one-carbon unit is activated to 10-formyltetrahydrofolate via the synthetase activity of the trifunctional enzyme C_1-tetrahydrofolate (THF) synthase for use in purine synthesis or can be further reduced to 5,10-methylene-THF by the dehydrogenase activity of the same enzyme. 5,10-Methylene-THF is used by serine hydroxymethyltransferase (SHMT) in the synthesis of serine. Recently, ^{13}C NMR has been used to establish that the C_1-THF synthase/SHMT enzyme system is the only route from formate to serine *in vivo* in the yeast *Saccharomyces cerevisiae* [Pasternack et al. (1992) *Biochemistry 31*, 8713–8719]. *In vitro* studies have considered the kinetics of the C_1-THF synthase/SHMT enzyme system in the catalytic conversion of formate to serine [Strong et al. (1987) *J. Biol. Chem. 262*, 12519–12525]. In the present work, we begin to study the kinetics of this two-enzyme system in its natural enviroment. Provision of [^{13}C]formate and direct detection of an intracellular accumulating pool of [3-^{13}C]serine by ^{13}C NMR of whole cells allow us to monitor the rate of flux through this enzyme system *in vivo*. The rate of accumulation of soluble [3-^{13}C]serine under [^{13}C]formate-saturating conditions is $13.0 \pm 1.2\ \mu M$/min relative to an external standard of serine in D_2O. The extracellular formate concentration at half-maximal flux was determined to be 900 μM. Direct observation of the effect of THF depletion induced by exposure to methotrexate and sulfonilamide indicates folate cofactor availability to be the rate-limiting factor in this enzyme system *in vivo* under formate-saturating conditions. The profile of loss of activity of the C_1-THF synthase/SHMT enzyme system reveals a compartmentation of reduced folate pools available to thymidylate synthase and the C_1-THF synthase/SHMT enzyme system. Modeling of non-steady-state kinetics through C_1-THF synthase/SHMT predicts the effective local THF pool concentration to be less than 1.0 μM at C_1-THF synthase/SHMT *in vivo*.

Tetrahydrofolate (THF)[1]-mediated one-carbon metabolism is essential in many cellular processes including nucleic acid biosynthesis, mitochondrial and chloroplast protein biosynthesis, amino acid metabolism, and methyl group biogenesis (Blakley, 1969). In addition to one-carbon metabolism, the folate cofactor plays a role in such nonmetabolic processes as DNA repair (Sancar, 1990). The major source of one-carbon units in most organisms is the 3-carbon of serine, derived from glycolytic intermediates (Schirch, 1984). Serine is cleaved by serine hydroxymethyltransferase (SHMT) (Figure 1, reaction 4) to generate glycine and 5,10-methylene-THF (CH_2-THF). This form of the coenzyme is then distributed between several pathways, depending on the needs of the cell. Conversion of CH_2-THF to 10-formyl-THF (CHO-THF) for use in purine synthesis occurs via the trifunctional enzyme C_1-THF synthase (Figure 1, reactions 1–3). Generation of one-carbon units in this manner occurs in both the cytosol and mitochondria as isozymes of SHMT and C_1-THF synthase exist in both compartments (Zelikson & Luzzati, 1977; Shannon & Rabinowitz, 1986). In the reverse direction, formate has been shown to contribute to the one-carbon pool by activation to CHO-THF and conversion to CH_2-THF by C_1-THF synthase (McKenzie & Jones, 1977; Ogur et al., 1977). Indeed, in the yeast *Saccharomyces cerevisiae*, *ser1*$^-$ strains (blocked in the production of serine from glycolytic intermediates) can satisfy all their one-carbon requirements via this pathway (McKenzie & Jones, 1977; Appling & Rabinowitz, 1985; Barlowe & Appling, 1990).

The predominate metabolism of formate to serine is attributed to the cytosolic C_1-THF synthase/SHMT enzyme system. First, *ser1*$^-$ yeast strains lacking one or more of the activities of cC_1-THF synthase fail to grow on formate plus glycine (McKenzie & Jones, 1977; Barlowe & Appling, 1990) whereas deletion of the *MIS1* gene (encoding the mitochondrial C_1-THF synthase) does not affect growth on formate plus glycine (Shannon & Rabinowitz, 1988). In addition, the detection of label originating from formate to the third carbon of serine is *almost* completely lost in the absence of cC_1-THF synthase (Pasternack et al., 1992). Although these results point to the cytoplasmic pathway as the major route for formate assimilation, they do not rule out a contribution by the mitochondrial pathway. Indeed, assimilation by the mitochondrial C_1-THF synthase (mC_1-THF synthase) has been demonstrated (Pasternack et al., 1992).

[†] This work is supported by an NIH Grant to D.R.A. and by NSF CHE 9013384 to D.A.L. L.B.P. was supported by a predoctoral fellowship from the Department of Education.

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® Abstract published in *Advance ACS Abstracts*, May 15, 1994.

[1] Abbreviations: CH_2-THF, 5,10-methylene-THF; CHO-THF, 10-formyl-THF; CH^+-THF, 5,10-methenyl-THF; cC_1-THF synthase, cytosolic C_1-tetrahydrofolate synthase; GCV, glycine cleavage enzyme system; dTMP, deoxythymidine monophosphate; DHFR, dihydrofolate reductase; MTX, methotrexate; mC_1-THF synthase, mitochondrial C_1-tetrahydrofolate synthase; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase.

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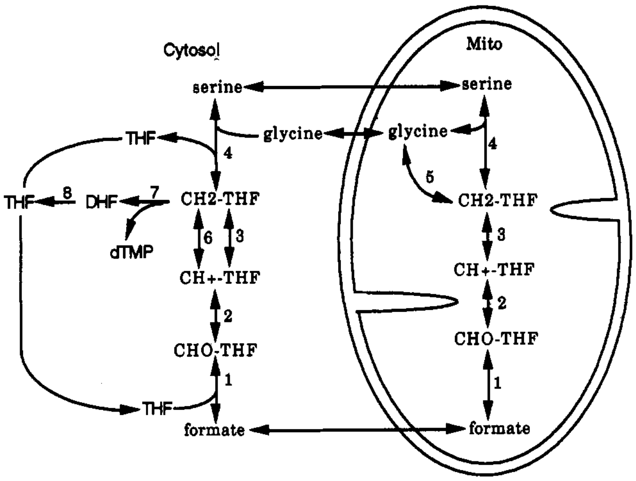

FIGURE 1: Proposed organization of the enzymes of single-carbon metabolism in yeast. Reactions 1–3, 10-formyl-THF synthetase (EC 6.3.4.3), 5,10-methenyl-THF cyclohydrolase (EC 3.5.4.9), and 5,10-methylene-THF dehydrogenase (EC 1.5.1.5), respectively, are catalyzed by the trifunctional C$_1$-THF synthase, encoded by the *ADE3* gene (Jones, 1972; Staben & Rabinowitz, 1986) for the cytosolic enzyme and the *MIS1* gene (Shannon & Rabinowitz, 1986, 1988) for the mitochondrial enzyme. Reaction 4 is SHMT (EC 2.1.2.1). Reaction 5 is the glycine cleavage enzyme system (EC 2.1.2.10) and is found only in the mitochondria. Reaction 6 is NAD-dependent 5,10-methylene-THF dehydrogenase. Reaction 7 is thymidylate synthase (EC 2.1.1.45), and reaction 8 is dihydrofolate reductase (EC 1.5.1.3).

In vitro kinetic analysis of both C$_1$-THF synthase and SHMT has provided information on the kinetic constants of these enzymes. The two-enzyme system has been reconstituted *in vitro* in a coupled reaction in which formate is converted to serine with regeneration of the THF used in the initial activation of formate (Strong et al., 1987). Thus, THF is used catalytically within the two-enzyme system. These authors determined that the synthetase activity of the C$_1$-THF synthase is the rate-limiting enzymatic step in the coupled enzyme system. The K_m for formate is shown to be dependent on the state of polyglutamylation and ranges from 2 mM to 4 μM for the monoglutamate and triglutamate forms, respectively. In addition, increasing concentrations of SHMT resulted in an increase in the activity of the synthase (Strong & Schirch, 1989). These authors suggest that, in the case of C$_1$-THF synthase and SHMT, protein–protein association may act to increase catalytic rates of the synthase. There is increasing evidence that, in the intact cell, many metabolic processes are organized into multienzyme complexes. Thus, cellular conditions might be expected to cause *in vivo* catalytic rates to be quite different from the rates measured *in vitro* of the isolated enzymes. Investigation of catalytic activity *in vivo* has been limited, however, due to lack of appropriate direct detection methods.

As described above, THF is a critical cofactor in the C$_1$-THF synthase/SHMT enzyme system. In higher eukaryotes the major source of THF is dietary intake of folic acid while in *S. cerevisiae* both exogenous folic acid and *de novo* synthesis contribute to the THF pool (Iwai et al., 1977). Dihydrofolate reductase (DHFR) is a critical enzyme in the maintenance of THF pools in several ways. First, *de novo* production of THF initially involves synthesis of dihydrofolate from guanosine and *p*-aminobenzoate with subsequent reduction to THF by DHFR (Shiota, 1984). Second, exogenous folic acid must be reduced to THF by DHFR prior to use. Furthermore, in rapidly dividing cells, synthesis of thymidine monophosphate (dTMP) by thymidylate synthase (TS) (Figure 1, reaction 7) results in a considerable drain on folate pools (Blakley, 1969; Shane & Stokstad, 1985). TS is the only folate-dependent enzyme that does not regenerate the reduced form of the cofactor after one-carbon transfer. Instead, DHF is generated by the reaction and must be reduced to THF by DHFR for the maintenance of cellular THF pools. DHFR is thus a critical enzyme in rapidly dividing cells and is a favorite target of anticancer drug therapy. Exposure to antifolates such as methotrexate (a competitive inhibitor of DHFR) results in an inability to regenerate THF for carrying one-carbon units. Studies in L1210 leukemia cells demonstrate that THF depletion occurs quickly ($t_{1/2}$ = 1.5 min) upon exposure to antifolates such as methotrexate and trimetrexate, but not completely (only 27% of the THF pool is oxidized to DHF) (Seither et al., 1989). These authors suggest that "a fraction of cellular tetrahydrofolates are in a biochemical form, physical compartment, or bound to cellular constituents which make them unavailable for oxidation to dihydrofolate". Folate depletion in *S. cerevisiae* requires inhibition of the *de novo* pathway in addition to inhibition of DHFR. Sulfonilamide inhibits *de novo* synthesis by substrate competition with *p*-aminobenzoate at dihydropteroate synthase (Brown, 1962).

In the present work, we have used *in vivo* ^{13}C metabolic labeling and direct ^{13}C NMR detection of intact cells to start to investigate metabolic flux through the cytoplasmic C$_1$-THF synthase/SHMT enzyme system in its natural enviroment. This system offers a unique opportunity to study the rate of this reaction *in vivo*, within its natural compartment, in association with other enzymes. This is accomplished by exogenous provision of saturating concentrations of [^{13}C]-formate and detection of the product of the pathway, [3-^{13}C]-serine. This kind of study requires careful quantitation of an accumulating intracellular [3-^{13}C]serine pool and is difficult to accomplish using previously available methods. ^{13}C NMR of whole cells is, however, good at detecting small changes in intracellular concentration of labeled metabolites provided they accumulate in a soluble pool. Applying these methods to growing cultures of *S. cerevisiae*, we are able to observe a linear rate of serine accumulation under various *in vivo* conditions. Using this process as a probe of the activity of the C$_1$-THF synthase/SHMT enzyme couple, we can begin to evaluate kinetic parameters of the reaction *in vivo*. Exposure of cells to the antifolates MTX and sulfonilamide prior to labeling with [^{13}C]formate reveals THF availability as the rate-limiting factor in flux through the C$_1$-THF synthase/SHMT enzyme system under these conditions. In addition, the pattern of loss of activity of these enzymes during antifolate exposure indicates differential compartmentation of the reduced folate pools available to thymidylate synthase and C$_1$-THF synthase/SHMT in yeast, analogous to observations in L1210 cells regarding enzymes of purine synthesis (Seither et al., 1989). An initial kinetic model of the *in vivo* flux through C$_1$-THF synthase/SHMT is presented.

EXPERIMENTAL PROCEDURES

Materials and Strains. Haploid strains of *S. cerevisiae* were used in all studies. Yeast strains DAY4 (**a** *ADE3 MIS1 ser1 ura3-52 trp1 leu2 his4*) and DAY3 (**a** *ade3-130 MIS1 ser1 ura3-52 trp1 leu2*) have been previously described (West et al., 1993). [^{13}C]Formate was purchased from Cambridge Isotope Laboratories (Woburn, MA).

Cell Growth and ^{13}C Labeling. Yeast cultures, grown aerobically at 30 °C in rich media (1% yeast extract, 2% bactopeptone, 2% glucose), were harvested at midlog phase by centrifugation at 4000 rpm for 4 min and resuspended to 37% w/v in yeast minimal media (yeast nitrogen base) containing 2% glucose, L-leucine (30 mg/L), L-histidine (20 mg/L), L-tryptophan (20 mg/L), uracil (20 mg/L), adenine (20 mg/L), and glycine (20 mg/L). Cultures were incubated aerobically (rpm = 250) at 30 °C and provided with labeled [^{13}C]formate at concentrations and for times as indicated in the figure legends.

Analysis of Extracts. After incubation with [^{13}C]formate as described above, cultures were centrifuged at 4000 rpm for 4 min, washed with 25 mL of 1.8 mM KH_2PO_4/118 mM NaCl (pH 7.4), and then resuspended in 1.8 mL of the same buffer containing 4 M 2-mercaptoethanol (in order to maintain reduction of folate derivatives). Cells were disrupted by vortexing for 4 min with glass beads, boiled for 5 min, and centrifuged for 25 min at 25000*g*. A portion of the resulting supernatant was transferred to a 5-mm NMR tube and overlaid with argon prior to capping. ^{13}C NMR spectra were obtained on a Nicolet Analytical Instruments NT 360 equipped with a 5-mm probe tuned to 90 MHz. A pulse width corresponding to a 45° flip angle was used, and data were collected with a 200-ms delay and continuous broad-band decoupling. A total of 3600 scans of 64K data points were acquired over a bandwidth of 20 000 Hz. Free induction decays were processed with base-line correction, line broadening of 3.0, and a zero fill prior to Fourier transform. Relative [3-^{13}C]serine concentrations in extracts are determined by comparison to an internal standard, the C2 resonance of glutamic acid. This assumes that the glutamic acid concentration is the same in the two strains. Comparison of signal intensities between many extracts indicates a variation of less than 20%.

Whole-Cell Analysis. Yeast cultures were exposed to [^{13}C]-formate as described above. Twenty-five-milliliter aliquots were removed at the appropriate time, frozen in methanol/dry ice, and stored at −70 °C. Just prior to NMR analysis, samples were thawed at 4 °C, centrifuged cold for 4 min at 4000 rpm, resuspended in 1 mL of D_2O, and transferred to 10-mm NMR tubes. This provided a total volume of approximately 7 mL of cell suspension. Cells were allowed to settle for 7 h at 4 °C prior to data aquisition. This resulted in a cell volume that exceeded the active probe volume and ensured that each spectrum was acquired from the same volume of cells. Incubation media were saved for analysis when necessary. ^{13}C NMR spectra were obtained as described above with the following changes. ^{13}C NMR spectra were acquired using a 10-mm probe. 1800 scans of 16K data points were aquired over an 11 904-Hz bandwidth. Pulse sequence parameters included a 45° flip angle and 1.5-s delay time. Comparison of peak areas for the resonance associated with the third carbon of serine at a 1.5 vs 5.0 s delay ($>4T_1$) revealed quantitative spectra would be acquired at an abbreviated delay time by reducing the flip angle. For each experiment, all samples and a standard curve of serine in D_2O (external standard) were run consecutively with the same pulse conditions and receiver gain. In order to accommodate day-to-day variation in NMR performance, integrated values for serine resonances were extrapolated from the standard curve run with each data set. No adjustments were made for differences such as T_1 typical of external standards, and thus values obtained in this manner, while accurately reflecting relative changes in concentration, may not reflect actual concentrations. NMR peak integration was performed by using NIH Image software (Russ, 1992). Graphical data are presented as the extrapolated values. Final enzyme flux values are presented as the intracellular concentration, C_{int}, by the following conversion:

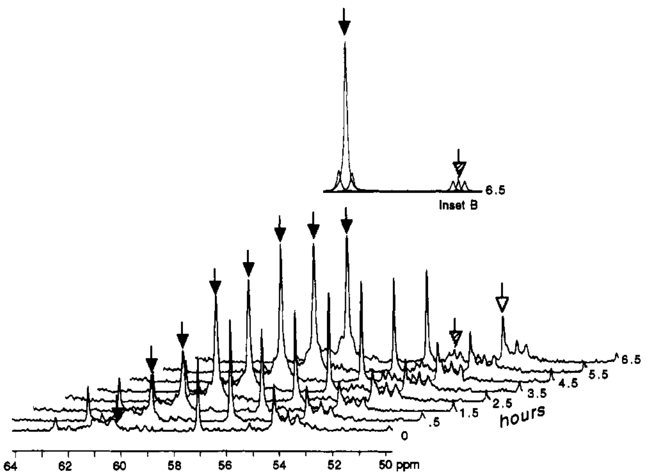

FIGURE 2: Whole-cell ^{13}C NMR spectra of [3-^{13}C]serine accumulation in strain DAY4 exposed to [^{13}C]formate. Each spectrum is identified by the number of hours of [^{13}C]formate exposure. Inset B is the deconvolution of the C3 and C2 serine resonances after 6.5-h exposure. The filled arrow indicates C3 serine resonance. The hatched arrow indicates C2 serine resonances. The open arrow indicates C4 glutamate resonance.

$$C_{\text{int}} = C_{\text{total}}/0.75$$

C_{total} represents the value obtained from standard curve. Division by 0.75 corrects for the water content of yeast cells of 75% (Kockova-Kratochvilova, 1990). Thus, with the yeast cells "packed" in the NMR tube, only 75% of the volume within the NMR coil is actually intracellular water space.

HMQC Detection of [3-^{13}C]Serine Resonance in Whole Cells. A ^{13}C–^{1}H correlated heteronuclear multiple quantum coherence spectrum of whole cells of yeast strain DAY4 exposed to [^{13}C]formate for 55 min was acquired on a Bruker AMX 500 with a $1/(2J)$ delay of 2.6 ms (Bax et al., 1983). A total of 256 blocks of 1024 points was acquired with 8 scans per block, for a total acquisition time of 2 h. Bandwidths were 6097 and 25 000 Hz for ^{1}H and ^{13}C NMR, respectively.

RESULTS

Whole-Cell Detection of Metabolism of [^{13}C]Formate to [3-^{13}C]Serine and [2-^{13}C]Serine. The ability of ^{13}C NMR to distinguish which carbon(s) in a particular metabolite is (are) labeled permits the appearance of label at the C3 position in serine to be directly attributed to the cytoplasmic C_1-THF synthase/SHMT system. This is important because the mitochondrial metabolism of formate was shown to result in label incorporation at the C2 and C3 position of serine (Pasternack et al., 1992). This is seen when a culture of strain DAY4, wild-type for both cytoplasmic and mitochondrial C_1-THF synthase, was incubated with [^{13}C]formate. Samples were removed at 30 min after introduction of formate and every subsequent hour. The spectra obtained (Figure 2) show that concentrations of [3-^{13}C]serine in the cell population increase with time. No significant NMR signal appears at this chemical shift in the absence of [^{13}C]formate (Figure 2; $t = 0$). Typical resonances associated with metabolism of [3-^{13}C]serine to [2-^{13}C]glycine can be seen at about the fourth hour as label starts to appear at the C2 position of serine. Both the single resonance of [2-^{13}C]serine and the doublet associated with the C–C coupling of [2,3-^{13}C]serine can be seen in these spectra. This is proposed to occur as mitochondrial [^{13}C]-CH_2-THF is converted to [2-^{13}C]glycine by the glycine cleavage system (GCV) (Figure 1, reaction 5). With time, the glycine pool used for serine synthesis becomes more and more labeled, resulting in appearance of label at the C2 position of serine. [2-^{13}C]Glycine itself is apparent in the spectra at 41.7 ppm (not shown) and accumulates over time in approximately a 1:3 relationship with [2,3-^{13}C]serine. Deconvolution and integration of these resonances indicate that the maximum accumulation of [3-^{13}C]serine occurs at about 5 h. Subsequent analysis of media [^{13}C]formate levels indicated that sufficient [^{13}C]formate remains in the media after 4 h to continue saturating the reaction.

Identification of [3-^{13}C]Serine in Whole-Cell Spectrum. Identification of resonances in whole-cell spectra can be difficult because the standard method for identifying a peak requires addition of a known compound to the sample. Although this can be accomplished in extracts, it is not possible in whole-cell measurements. Identification of resonances associated with serine in extracts was previously reported (Pasternack et al., 1992). ^{13}C spectrum of extracts of cells exposed to [^{13}C]formate for 3 h (Figure 3B) and ^{13}C spectrum of whole cells exposed to [^{13}C]formate for 6.5 h (Figure 2; t = 6.5 h) are similar, which suggests that the resonances have the same chemical shifts in whole cell as in the extract. This identification was confirmed by a HMQC experiment in which a 2D map was generated that correlates the carbon chemical shift with the proton chemical shift (data not shown).

Consideration of Alternate Sources and Loss of [3-^{13}C]-Serine Signal in Vivo. Typically, *in vitro* kinetic measurements are followed by the accumulation of product of a reaction in a closed system where loss of the detectable product is not a factor. *In vivo*, however, measurement of an accumulating end product is subject to (1) gain in signal due to alternate routes of synthesis and (2) loss of signal due to exodus from intermediate or final metabolite pools. These two points are considered in the following studies. Strains DAY3 (lacking cC$_1$-THF synthase) and DAY4 (wild-type) were incubated for 3 h with [^{13}C]formate under well-aerated conditions in order to determine the extent to which mitochondrial assimilation of formate contributes to the accumulation of [3-^{13}C]serine. An extract was prepared as described in Experimental Procedures. The spectrum in Figure 3A shows the largest amount of [3-^{13}C]serine detected of three experiments performed in strain DAY3. The integral of 3-serine in spectra of both strains is normalized to the 2C resonance of glutamic acid (not derived from one-carbon metabolism) as described in Experimental Procedures. Comparison of the total [3-^{13}C]serine present in extracts of strains DAY4 and DAY3 under these aerating incubation conditions indicates a maximum of 6.2 ± 1.7% due to mitochondrial metabolism of [^{13}C]formate. Thus the accumulating [3-^{13}C]serine pool in strain DAY4 can be thought of as a contribution of 94% from flux through cC$_1$-THF synthase and 6% from flux through mC$_1$-THF synthase. Spectra from strains carrying deletions of both mC$_1$-THF synthase and cC$_1$-THF synthase show no accumulation of [3-^{13}C]serine upon incubation with [^{13}C]formate under aerating conditions (Pasternack et al., 1992).

Exodus from the pool can be estimated by incorporation of [^{13}C]CH$_2$-THF into glycine. Evaluation of the appearance of [2-^{13}C]serine (singlet at C2 serine) and [2,3-^{13}C]serine (doublet at C2 serine and C3 serine) in the extract of strain DAY4 provides an indication of the use of [^{13}C]CH$_2$-THF for glycine synthesis. Synthesis of glycine is evident in these extracts at 3 h. In whole-cell detection, no [2-^{13}C]serine or [2,3-^{13}C]serine is evident until after 3.5 h (Figure 2, t = 3.5 h). Thus whole-cell studies are limited to the first 90 min of formate exposure in order to minimize the effect of [2,3-^{13}C]serine accumulation on the integration of the [3-^{13}C]-resonances.

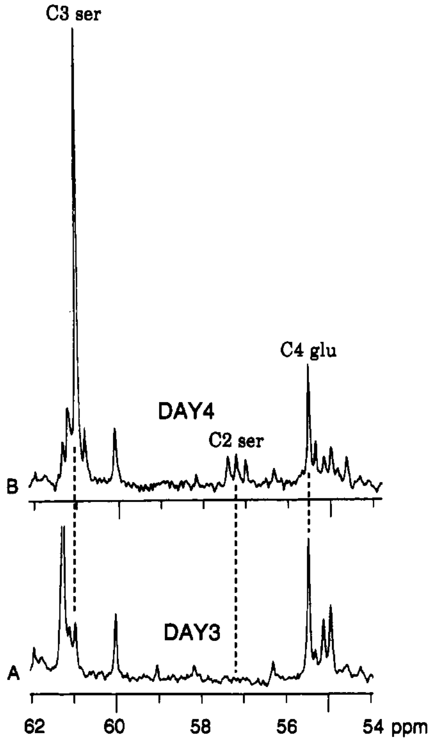

FIGURE 3: ^{13}C NMR of extracts of strains (A) DAY3 (lacks cC$_1$-THF synthase) and (B) DAY4. Cultures were exposed to [^{13}C]formate for 3 h prior to extraction.

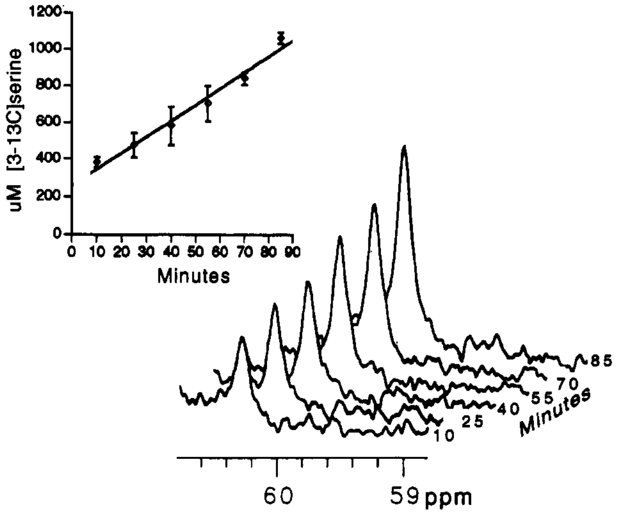

FIGURE 4: ^{13}C NMR spectra of [3-^{13}C]serine in whole cells exposed to [^{13}C]formate for varying times. Each spectrum is identified by the time exposure to [^{13}C]formate. The graph depicts values obtained from three experiments. Error bars are means ± SEM.

Determination of [3-^{13}C]Serine Accumulation Rate during Initial Hours. Strain DAY4, wild-type for both cytoplasmic and mitochondrial C$_1$-THF synthase, was incubated with [^{13}C]-formate and prepared for whole-cell analysis as described above. Samples were removed at 10 min and every subsequent 15-min interval after introduction of [^{13}C]formate. The spectra displayed in Figure 4 are from one of three experiments performed. Data for all three experiments are combined on the graph (Figure 4). The overall rate of accumulation of [3-^{13}C]serine with increasing time is linear between 10 and 85 min. Whole-cell spectra were not subjected to background

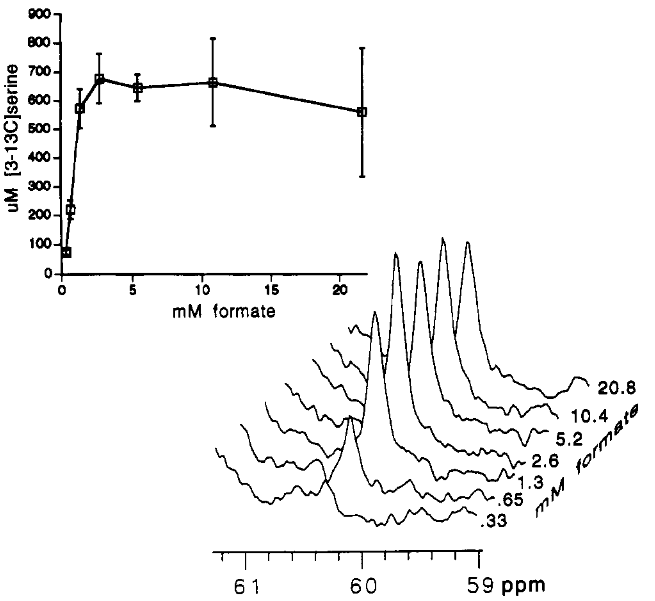

FIGURE 5: ^{13}C NMR spectra of [3-^{13}C]serine in whole cells exposed to varying concentrations of [^{13}C]formate. Each spectrum is identified by the concentration of [^{13}C]formate. The graph depicts values obtained from three experiments. Error bars are the means ± SEM.

subtraction, which accounts for the line not passing through zero. Total rate of intracellular accumulation of [3-^{13}C]serine over the 1-h period was calculated to be 13.0 ± 1.2 μM/min relative to the external standard of serine in D_2O. Because the rate of accumulation is linear and reproducible between 10 and 85 min, additional experiments comparing rates by single time point are done by incubation with [^{13}C]formate for 55 min.

Effect of Formate Concentration on [3-^{13}C]Serine Accumulation. Strain DAY4 was incubated with varying concentrations of [^{13}C]formate and prepared for whole-cell analysis as described above. Cells were removed at 55 min after introduction of [^{13}C]formate. The spectra displayed in Figure 5 are from one of three experiments performed. Combined data from the three experiments are shown on the graph (Figure 5). The accumulation of [3-^{13}C]serine increased linearly with increasing formate concentrations up to 1.3 mM external formate (Figure 5). Above 2.6 mM formate, serine accumulation did not increase, indicating the pathway was saturated with respect to formate. The formate concentration that yields half-maximal metabolic flux is approximately 900 μM. For all other experiments, a saturating concentration of 5.2 mM exogenous formate was used.

Growth of Yeast Exposed to Methotrexate/Sulfonilamide. In order to study the effect of THF concentration on the rate of the C_1-THF synthase/SHMT enzyme system, yeast cultures were incubated with antifolates. Cultures of strain DAY4 grown in rich media were exposed to 25 mg/L MTX and 5 g/L sulfonilamide in early log phase as indicated, and growth was monitored (Figure 6). The effect on growth in rich media is not apparent until several doubling times (one doubling time is approximately 1.5 h) after addition of the drug. These results compare with previously published results (Kunz et al., 1986). Inability of the drug-treated culture to achieve final density is repeatable and more pronounced in minimal media (data not shown). A decrease in growth rate is not detectable until at least 6 h of exposure to the drug. This provides a time frame in which the effect of THF depletion can be studied in rapidly dividing cells.

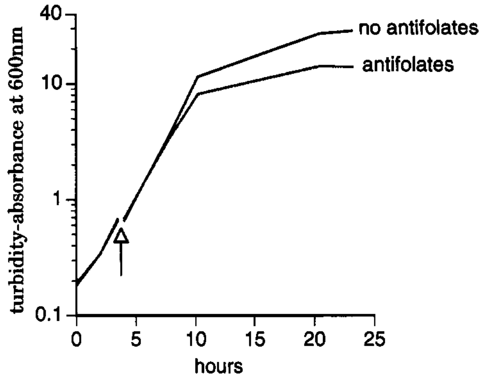

FIGURE 6: Effect of MTX/sulfonilamide on growth of strain DAY4. Cells growing logarithmically in rich media were treated with or without drugs (25 mg/L MTX and 5 g/L sulfonilamide) at the time indicated by the arrow, and growth was monitored by turbidity at A_{600}. No decrease in growth rate is observed until 6 h after addition of antifolates.

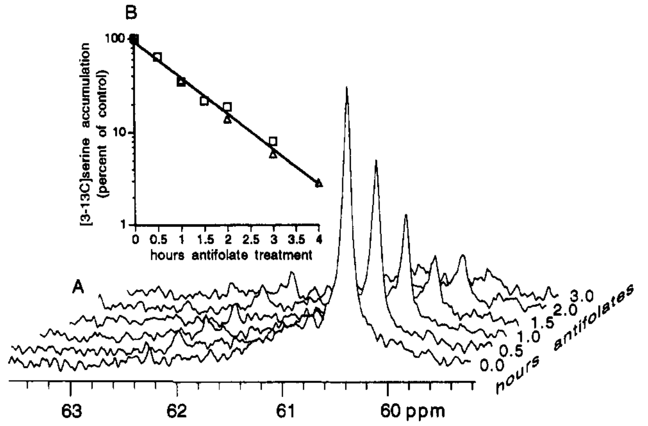

FIGURE 7: (A) ^{13}C NMR spectra of [3-^{13}C]serine in whole cells of strain DAY4 grown in rich media and preexposed to antifolates prior to incubation with [^{13}C]formate. Each spectrum is identified by the preexposure time. (B) Graph depicts the percent [3-^{13}C]serine present relative to the [3-^{13}C]serine present in cells not treated with antifolates (no drug control). [3-^{13}C]Serine resonance as detected in whole cells (□) and with extracts prepared prior to NMR analysis (△) is indicated.

Exposure to Antifolates Reduces Flux through the THF-Dependent C_1-THF Synthase/SHMT Enzyme System. Strain DAY4 was grown in rich media with exposure to MTX and sulfonilamide for various times. Cells were harvested, resuspended to 37% w/v in fresh minimal medium lacking antifolates, and then incubated with [^{13}C]formate for 55 min. Whole-cell spectra were acquired, and concentration of [3-^{13}C]serine was calculated as described. Direct *in vivo* detection of [3-^{13}C]serine indicates that a decreasing flux through the C_1-THF synthase/SHMT enzyme system occurs with increasing time exposure to the drugs during log growth (Figure 7A). No such loss in activity was detected when stationary-phase cells were incubated with antifolates prior to exposure to [^{13}C]formate (data not shown). Percent area of the [3-^{13}C]serine resonance relative to the area of the [3-^{13}C]serine resonance in the no drug control is plotted in Figure 7B. Curve fitting indicates that the data are best described by a simple exponential decrease (Figure 7B, □), with no lag in inhibition. In order to verify this, the experiment was repeated in greater detail by lengthening the exposure time of MTX/sulfonilamide to 4 h, exposure to [^{13}C]formate for 3 h, and preparation of extracts. The relative concentration of [3-^{13}C]serine was determined by integration and normalization to the C2 resonance of glutamic acid. The extract data (Figure 7B, △) display the same exponential loss of activity of the synthase as seen in the whole-cell direct detection study.

DISCUSSION

C_1-THF synthase is considered to be the primary route of assimilation of formate in eukaryotes. Yeast strains that lack both cytosolic and mitochondrial isozymes of C_1-THF synthase were unable to accumulate ^{14}C label following exposure to [^{14}C]formate (Barlowe & Appling, 1990). In another study, accumulation of [3-^{13}C]serine in yeast cells exposed to [^{13}C]-formate was directly attributed to the presence of C_1-THF synthase (Pasternack et al., 1992). Although both cytosolic and mitochondrial assimilation are apparent, lower aeration conditions are required for the accumulation of [3-^{13}C]serine due to mitochondrial assimilation of [^{13}C]formate. Thus high aeration permits targeting of formate metabolism to the cytoplasmic pathway. Still, about 6% of the serine accumulation seen in these *in vivo* studies may be attributed to mitochondrial assimilation of formate.

Intracellular [3-^{13}C]serine accumulates at the rate of 13.0 ± 1.2 μM/min during the first 90 min of exposure to [^{13}C]-formate (Figure 4), and maximum accumulation of [3-^{13}C]-serine is achieved at about 5-h exposure to [^{13}C]formate (Figure 2). To put this in perspective in terms of the cell, this is an accumulation of about 4 mM [3-^{13}C]serine and compares with reported intracellular serine concentrations of 2–5 mM (Jones & Fink, 1982). Thus, complete turnover of the serine pool occurs in 5 h. The calculated rate of 13 μM/min represents a *minimum* flux through C_1-THF synthase/SHMT since exodus from intermediate pools and the serine pool itself *in vivo* is not yet entirely defined. Keeping in mind the 10–20% percent uncertainty inherent in the method, comparison of the signal to an external standard curve allows us to monitor the change in the rate of accumulation of [3-^{13}C]serine and thus begin to evaluate kinetic parameters of the reaction *in vivo*. Since the synthetase activity of C_1-THF synthase has been shown to be the rate-limiting step in an *in vitro* coupled C_1-THF synthase/SHMT system (Strong et al., 1987), variation in flux as detected by accumulation of [3-^{13}C]serine is assumed to reflect variation in synthetase activity *in vivo*.

The effect of increasing formate concentration on rate of flux through C_1-THF synthase/SHMT was determined. The accumulation of [3-^{13}C]serine increased linearly with increasing formate concentrations up to 1.3 mM external formate (Figure 5). Above 2.6 mM formate, serine accumulation did not increase, indicating the pathway was saturated with respect to formate. It should be noted that the highest concentration tested (20.8 mM) is the concentration typically used for yeast growth studies. This concentration consistently resulted in inhibition of the overall flux to serine synthesis. The media formate concentration at half-maximum serine synthesis is 900 μM. Typical K_m values for formate for the 10-formyl-THF synthetase activity of C_1-THF synthase range from 4 μM to 2.5 mM, depending on the state of polyglutamylation of the THF cofactor (Strong et al., 1987). It should be noted that *in vitro* K_m determinations for formate are done at saturating THF levels. As described below, *in vivo* conditions are almost certainly not saturating for THF.

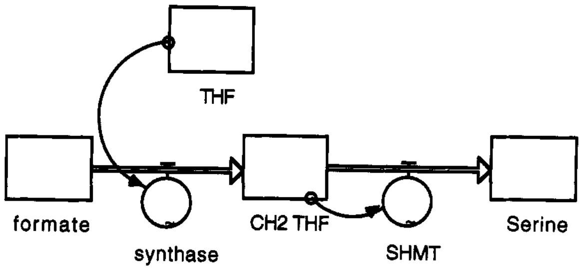

FIGURE 8: Main chain of STELLA model of rate of flux through C_1-THF synthase/SHMT. Rate of enzyme activities is determined by kinetic parameters as described in Table 1.

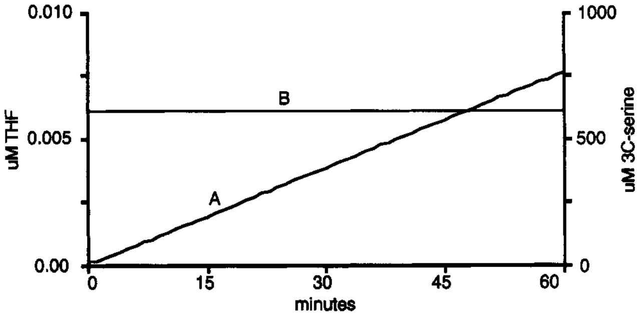

FIGURE 9: STELLA simulation of the metabolic flux of formate to serine during the first hour. (A) Serine accumulation. (B) THF concentration throughout simulation.

The dependence of the flux through this system on THF levels was studied by exposure of the cells to MTX/sulfonilamide during log growth. Increasing time exposure to antifolates during exponential growth resulted in decreased [3-^{13}C]serine accumulation upon exposure to [^{13}C]formate. Curve fitting revealed that the data are best described as exponential (Figure 7) with no lag in inhibition. This suggests that THF pool availability is the rate-limiting factor in flux through C_1-THF synthase/SHMT *in vivo* under formate-saturating conditions. Thus a model can be built to describe the metabolic flux through the two-enzyme system based on the availability of THF (Figure 8). The STELLA modeling system was chosen as it provides the ability to define the rate of a reaction in the model based on typical enzyme kinetic parameters (Hayton & Collins, 1991; Hargrove, 1993). Each square represents a metabolic intermediate and each circle an enzyme. This is a kinetic (not steady-state) model since it attempts to describe the initial forward rate of the reaction as expected under substrate-saturating conditions. The basic kinetic model is comprised of C_1-THF synthase, SHMT, and the THF pool. The rate of each enzyme in the model is based on *in vitro* determined kinetic parameters of the THF cofactor (Table 1). The K_m and V_{max} for the polyglutamylated form of the cofactor are used whenever available, in order to better mimic the expected state of glutamylation of the cofactor in the cell. This model produces a linear response of serine accumulation with time (Figure 9, line A) when THF concentration (line B) is held constant, similar to what was observed experimentally (Figure 4). In agreement with *in vitro* studies that show the synthetase activity to be rate limiting in the coupled C_1-THF synthase/SHMT reaction, the synthetase activity is the rate-limiting enzyme activity in the kinetic model. The effect of exponentially decreasing THF pools on the rate of [3-^{13}C]serine production (SHMT activity) is simulated in Figure 10. The model predicts that the local THF concentration (i.e., that available to the active site of the rate-limiting enzyme) at which the cofactor becomes limiting for the reaction is approximately 1.0 μM. The local THF concentration must be lowered to 0.006 μM to obtain the experimentally observed flux through the C_1-THF synthase/SHMT system (as determined by [3-^{13}C]serine accumulation) (Figure 9). This predicted value is 3 orders of magnitude lower than reported intracellular THF concentrations. Total reduced folate concentrations have been determined in several different cell types, including the yeast *S. cerevisiae*, and range from 10 to 40 μM (Lor & Cossins, 1972; Cichowicz & Shane, 1987). This difference could be due to *in vivo* conditions (such as protein–protein interactions) that alter the affinity

Table 1: Kinetic Parameters Used in STELLA Model[a]

enzyme reaction	K_m (μM)	V_{max} (μmol/min L)	k_{cat} (min^{-1})	[E] (μM)	ref
C_1-THF synthase–synthetase	0.1	246[b]	82	6	Strong & Schirch, 1989
cSHMT glycine to serine		17 640[b]	980	18	Strong & Schirch, 1989
glycine to serine	0.4				Stover & Schirch, 1992
thymidylate synthase	7	331[c]	73	4.5[c]	Bisson & Thorner, 1981

[a] K_m values determined with the pentaglutamated form of the cofactor are used in this simulation in order to best reflect the glutamylation state of the cell environment. V_{max} and [E] values reflect calculated intracellular values. [b] V_{max} is calculated from k_{cat}[E]. [c] Calculated from purification table (Bisson & Thorner, 1981).

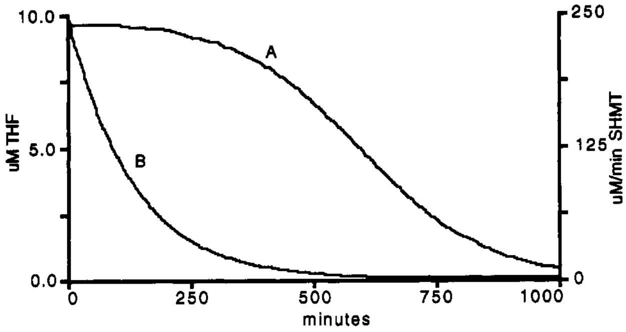

FIGURE 10: Simulated rate of SHMT with decreasing THF concentrations. (A) Rate of SHMT in main frame of STELLA simulation with decreasing THF concentrations; (B) concentration of THF throughout simulation. THF concentration is decreased exponentially over time as would occur with dilution due to exponential cell division.

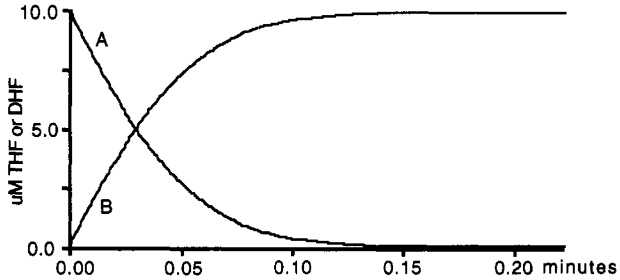

FIGURE 11: STELLA simulation of the conversion of THF to DHF with entire pool of reduced folates exposed to thymidylate synthase when DHFR is inhibited by antifolates. (A) THF; (B) DHF. The entire pool of THF is lost within the first minute.

of the enzyme for the cofactor. Alternatively, THF may be compartmentalized such that not all of the pool is available to the C_1-THF synthase/SHMT enzyme system *in vivo*.

Compartmentalization of THF pools is also suggested by the discrepancy between expected and observed rates of the THF-dependent reactions when antifolates are introduced into the kinetic model. Published kinetic values for the yeast TS indicate the capacity to oxidize the entire THF pool within seconds (Figure 11). Experimentally, however, loss of activity for C_1-THF synthase/SHMT is seen only during log growth and requires hours of exposure to antifolates (Figure 7). This indicates that the THF pool available to TS is somehow compartmentalized from the THF pool available to C_1-THF synthase/SHMT for serine synthesis. Seither et al. (1989) reached a similar conclusion in their studies with L1210 leukemia cells. THF loss upon antifolate treatment was only 28% of the total pool even though, again, kinetic parameters of TS predict complete loss of all available THF. The authors suggested that a major portion (72%) of the total cellular THF is unavailable for oxidation by TS. In those studies, label incorporation into purines ceased almost immediately even though THF pools persisted, suggesting that purine synthesis used the same pool of cofactor as TS. Studies presented here, however, show activity of C_1-THF synthase even after exposure to antifolates, indicating the availability of THF for the reaction. One could envision that the purine machinery (including C_1-THF synthase) and TS share the same THF pool, with serine synthesis involving C_1-THF synthase using a different pool. This would imply two populations of cytosolic C_1-THF synthases, one associated with purine synthesis and one associated with serine synthesis. This compartmentation could easily be accomplished through the formation of enzyme complexes. In fact, we have previously presented molecular genetic evidence for the presence of a purine enzyme complex involving C_1-THF synthase in yeast (Barlowe & Appling, 1990).

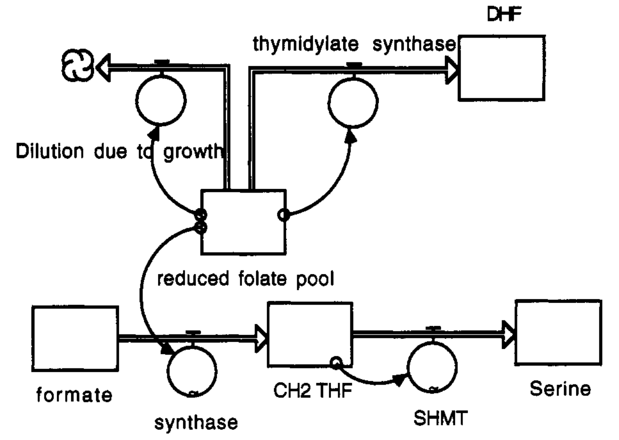

FIGURE 12: STELLA model of THF depletion during antifolate exposure in log-phase yeast cells. The model includes dilution due to log-phase growth and restricted exposure of the reduced folate pool to thymidylate synthase.

Since exposure to antifolates during log phase decreases C_1-THF synthase/SHMT activity while exposure during stationary phase does not, loss of THF pools can be attributed to cell division. However, exponential dilution of the THF pool in the kinetic model does not fully account for the decrease in [3-^{13}C]serine accumulation seen experimentally. We suggest that redistribution of reduced folate pools during cell division exposes additional folates to oxidation by thymidylate synthase. Figure 12 describes a model which includes (1) depletion of reduced folate pools due to cell division, i.e., an exponential dilution of the pool based on growth rate, and (2) limited exposure of reduced folates to thymidylate synthase. Figure 13 compares simulations with loss due to cell division alone (curve A) and with both cell division and thymidylate synthase activity (curve B). The thymidylate synthase contribution was determined by fit of the model to the experimentally observed rate of loss of serine synthesis during antifolate exposure (Figure 7).

One additional point deserves note. Studies of flux through C_1-THF synthase/SHMT during antifolate exposure were performed with [^{13}C]formate incubation for 1 h (Figure 7B, □) and for 3 h (Figure 7B, △) with the same percent loss of activity for the same time exposure to antifolates. If we

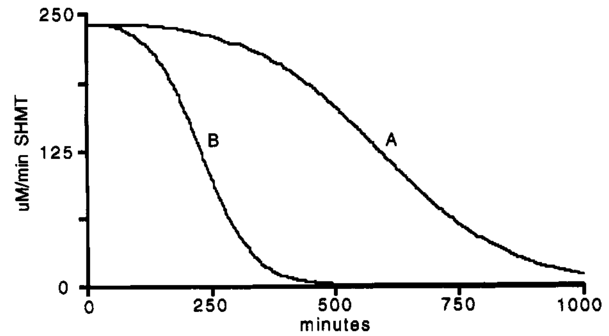

FIGURE 13: STELLA simulation of the effect of THF redistribution during cell growth on the rate of flux through C_1-THF synthase/SHMT enzyme system. The predicted profile of loss of enzyme activity with (A) dilution due to cell division only; (B) dilution due to cell division plus thymidylate synthase activity. Simulation was run starting with a 10.0 μM THF concentration.

consider total THF cellular concentrations to be 10 μM, and total production of [3-^{13}C]serine in a 1-h period to be 780 μM (60 min × 13 μM/min), it is easy to see that the THF used in the reaction is regenerated many times during the period of the hour. Under antifolate exposure, no additional loss of activity is seen at longer periods of incubation with [^{13}C]-formate. This suggests that the reduced folate pool remains constant over at least 3 h following drug treatment.

In the present work, we have followed the flux through the C_1-THF synthase/SHMT enzyme system in *S. cerevisiae* by ^{13}C NMR detection of the accumulation of [3-^{13}C]serine during exposure to [^{13}C]formate in whole cells. In the past, incorporation of [^{14}C]formate into [3-^{14}C]serine in human lymphocytes has been used as a diagnostic tool for folate deficiency (Ellegaard & Esmann, 1970). With the recent advances in spectroscopy and imaging (van Zijl et al., 1993), detection of [3-^{13}C]serine synthesis *in vivo* may provide a means to assess folate status in multiple tissues in a noninvasive manner. In addition, whole-cell detection of [3-^{13}C]serine by ^{13}C NMR coupled with the ease of genetic manipulation in yeast provides a unique opportunity to consider the enzymes in their natural environment. Although much information is available on the kinetics of this enzyme system *in vitro*, direct detection allows us to study the kinetics *in vivo*.

The question of how well *in vitro* determined kinetic parameters represent *in vivo* enzyme activity is difficult to address. The cellular environment includes protein–protein interactions, substrate channeling, local vs bulk precursor concentrations, and effectors still undefined. The work presented here begins to address the C_1-THF synthase/SHMT enzyme system in its natural environment in a manner comparable to *in vitro* enzyme kinetics. This work establishes a reproducible *in vivo* assay of flux through C_1-THF synthase/SHMT by direct detection of the end metabolite serine, evaluates the effects of formate and THF concentrations, and presents a dynamic model of flux through the enzyme system in order to aid in the evaluation of experimental data. In addition, the compartmentation of THF pools is revealed. Indeed, this system may provide an easily accessible non-mammalian eukaryotic model system for the study of the compartmentation of THF pools and the effects of antifolates in rapidly growing cells.

ACKNOWLEDGMENT

We would like to thank Dr. Dave Hoffman for guiding us through the HMQC experiment and Dr. John M. Beale, Jr., for many helpful discussions.

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