Characterization of the Folate-Dependent Mitochondrial Oxidation of Carbon 3 of Serine†

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ABSTRACT: The folate-dependent catabolism of serine was studied in intact rat liver mitochondria and soluble extracts from sonicated mitochondria. Formate and CO_2 are both known to be products of the mitochondrial oxidation of carbon 3 of serine. The present work tests the proposal [Barlowe, C. K., & Appling, D. R. (1988) *Biofactors 1*, 171–176] that carbon 3 of serine is first oxidized to 10-formyltetrahydrofolate, which can be either oxidized to CO_2 or converted to formate. Oxidation of carbon 3 of serine to formate and CO_2 was shown to be dependent on the respiratory state of the mitochondria. Formate production was greatest in state-3 (actively respiring) mitochondria and lowest in uncoupled mitochondria. In contrast, CO_2 production was greatest in uncoupled mitochondria and lowest in respiratory-inhibited mitochondria. Formate production appeared to be favored when high concentrations of $NADP^+$ and ADP were present, but there was no clear correlation between the $NADP^+$:NADPH redox state and CO_2 production. In soluble mitochondrial extracts, CO_2 production depended on $NADP^+$ and tetrahydrofolate, whereas formate production required ADP in addition to $NADP^+$ and the reduced folate cofactor. Unlike CO_2 production, however, formate production showed a complete dependance on a polyglutamylated form of the folate cofactor. These experiments support the proposed folate-mediated serine oxidation as a major pathway for the flux of one-carbon units through mitochondria.

In eukaryotes, the mitochondrial and cytosol compartments each contain a parallel set of one-carbon-unit interconverting enzymes (Appling, 1991). In the cytoplasm, the primary entry point of one-carbon units is 5,10-methylene-H_4folate,[1] derived from carbon 3 of serine via serine hydroxymethyltransferase (SHMT) (Figure 1, reaction 4) (Schirch, 1984). 5,10-Methylene-H_4folate can be oxidized to formate by the sequential action of $NADP^+$-dependent 5,10-methylene-H_4-folate dehydrogenase, 5,10-methenyl-H_4folate cyclohydrolase, and 10-formyl-H_4folate synthetase (Figure 1, reactions 3–1). In eukaryotes, these three activities are part of a trifunctional enzyme, C_1-tetrahydrofolate synthase. 10-Formyl-H_4folate can also be converted to CO_2 by the action of 10-formyl-H_4-folate dehydrogenase (Scrutton & Beis, 1979) (Figure 1, reaction 7). All of these activities are found in mitochondria as well (Barlowe & Appling, 1988, and references therein). Work in our laboratory has been directed toward understanding the respective role(s) of these two sets of isozymes in folate-mediated one-carbon metabolism.

We have proposed (Barlowe & Appling, 1988) an intercompartmental metabolic pathway in which serine, after transport into mitochondria, donates its 3-position carbon to H_4folate to generate a mitochondrial pool of 5,10-methylene-H_4folate. This one-carbon unit can be utilized for mitochondrial processes or oxidized to formate for transport out of the mitochondria and activation by cytoplasmic 10-formyl-H_4-folate synthetase. Initial experiments demonstrated that isolated rat liver mitochondria can oxidize carbon 3 of serine or the *N*-methyl carbon of sarcosine to formate, which rapidly exits the mitochondria (Barlowe & Appling, 1988). Although all of the activities of the proposed pathway (Figure 1, reactions 1–4) are localized in the mitochondrial matrix, the intact mitochondrial system used did not allow us to directly demonstrate the participation of the folate-dependent pathway. In this paper we describe the cofactor requirements of a mitochondrial extract catalyzing the oxidation of serine to formate and CO_2. In addition, experiments with isolated intact mitochondria reveal a dependence on the respiratory state of the mitochondria for this pathway.

EXPERIMENTAL PROCEDURES

Reagents. L-[3-^{14}C]Serine was purchased from either Amersham (Arlington Heights, IL) or ICN (Irvine, CA). $PteGlu_5$ was purchased from Dr. B. Schircks Laboratories (Jona, Switzerland). Dihydrofolate reductase (*Lactobacillus casei*) was purchased from Dr. Roy Kisliuk (Tufts University). Glucose-6-phosphate dehydrogenase (*Leuconostoc messenteroides*), formate dehydrogenase (*Pseudomonas oxalacticus*), and hexokinase (type IV) were purchased from Sigma (St. Louis, MO). All other chemicals were reagent grade. (6*RS*)-H_4folate was prepared by hydrogenation of folic acid over platinum oxide in a neutral aqueous solution (Blakley, 1957) and purified by chromatography on DEAE-cellulose (Curthoys & Rabinowitz, 1971). $PteGlu_5$ (10 mg) was reduced as described (Strong et al., 1989) and purified on a DEAE-Sephadex column according to Matthews et al. (1982). The reduced material was loaded using 20 mM K^+-BES (pH 7.0) and 20 mM 2-mercaptoethanol and eluted with a 50-mL linear gradient of the same buffer containing 0.6 M NaCl. Fractions (1 mL) were monitored for $H_4PteGlu_5$ by their absorbance at 294 nm and activity in a 10-formyl-H_4folate synthetase assay as described (Rabinowitz, 1985), except that yeast C_1-THF synthase was used. Peak fractions were pooled, lyoph-

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[1] Abbreviations: H_4folate or THF, tetrahydrofolate (unspecified polyglutamate chain length); $H_4PteGlu_n$, tetrahydropteroylpoly(γ-glutamate), with *n* indicating the number of glutamate moieties; BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; HEPES, *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SHMT, serine hydroxymethyltransferase; ACR, acceptor control ratio.

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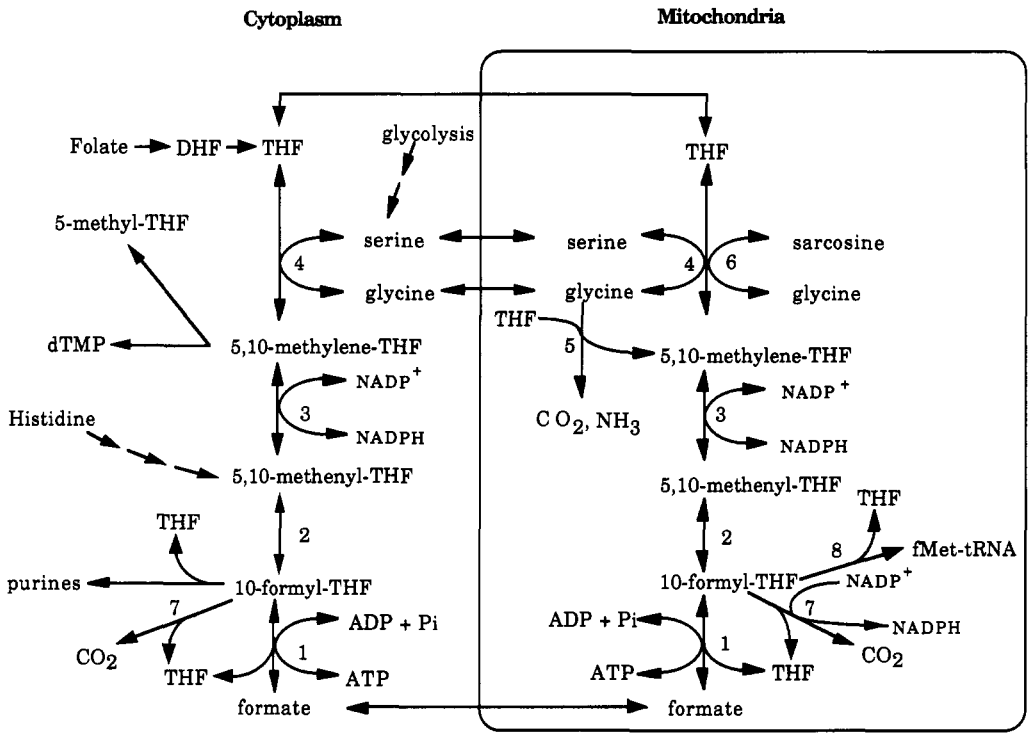

FIGURE 1: Compartmentation of folate-mediated one-carbon metabolism. Reactions 1, 2, and 3, 10-formyl-H_4folate synthetase (EC 6.3.4.3), 5,10-methenyl-H_4folate cyclohydrolase (EC 3.5.4.9), and 5,10-methylene-H_4folate dehydrogenase (EC 1.5.1.5), respectively, are catalyzed by C_1-tetrahydrofolate synthase. The other reactions are catalyzed by serine hydroxymethyltransferase (EC 2.1.2.1), reaction 4; glycine cleavage system (EC 2.1.2.10), reaction 5; sarcosine dehydrogenase (EC 1.5.99.1), reaction 6; 10-formyl-H_4folate dehydrogenase (EC 1.5.1.6), reaction 7; and methionyl-tRNA formyltransferase (EC 2.1.2.9), reaction 8. Not all coenzymes are shown for all reactions.

ilized, and resuspended in 0.01 M Tris-HCl (pH 7.0) and 0.1 M 2-mercaptoethanol. $H_4PteGlu_5$ was quantified by absorbance at 297 nm (Matthews et al., 1982) and by using the SHMT/5,10-methylene-THF dehydrogenase coupled assay (Stover & Schirch, 1992). SHMT was purified from *Escherichia coli* as described (Schirch et al., 1985). Yeast C_1-THF synthase was purified as described (Barlowe et al., 1989).

Mitochondria Preparations. Male Sprague-Dawley rats (200–300 g) were obtained from Harland–Sprague-Dawley (Houston, TX). Mitochondria from rat livers were prepared as described (Pedersen et al., 1978) except that 0.3 M sucrose, 1.0 mM EGTA, 5.0 mM MOPS, 5.0 mM K_2PO_4, and 0.1% BSA, adjusted to pH 7.4 with KOH, was employed as homogenization (H) medium (Rickwood et al., 1987). Acceptor control ratios (ACRs) of mitochondria were determined as described, using succinate as substrate (Pedersen et al., 1978). Protein determinations on whole mitochondria were done using the Biuret method (Bailey, 1967).

Washed mitochondria to be used for soluble crude extract studies were resuspended in H media without sucrose after the final wash. Protein concentration was adjusted to 13.5 mg/mL. Mitochondria were then sonicated for 3 min in 10-s bursts on ice, using a Vibra-Cell Model VC40 (Danbury, CT) at maximum power. The sonicated extract was centrifuged at 20000*g* for 30 min, and the supernatant fraction was dialyzed against H media minus sucrose.

Assays. Serine oxidation incubations contained 50 mM Hepes (pH 7.4), 50 mM KCl, 25 mM K_2HPO_4, 2 mM $MgCl_2$, and either intact mitochondria or sonicated mitochondrial supernatant in a total volume of 1.0 mL. Sucrose, 0.3 M final concentration, was included in reactions with intact mitochondria. Other additions are indicated in the figure legends. L-[3-^{14}C]Serine (2000 dpm/nmol) was used at 0.1 mM unless otherwise indicated. Reaction mixtures contained either 7.0 mg of intact mitochondrial protein in 0.4 mL of H medium or 0.4 mL of the sonicated mitochondrial supernatant. For intact mitochondria studies, O_2 was bubbled into the reaction buffer for 3 min prior to protein addition. Reactions were initiated by the addition of protein, and the reaction mixtures were placed in a 37 °C shaker bath. Parallel incubations were carried out for CO_2 and formate determinations. For CO_2 determinations in both intact and soluble systems, the incubations were carried out in Warburg flasks. To terminate the reaction and liberate the $^{14}CO_2$, 0.5 mL of 1.2 N perchloric acid was added from the side arm of the flask. Flasks were incubated at 37 °C for 1 h for CO_2 trapping, using 150 μL of phenethylamine in the center well of the Warburg flasks. One hundred microliters of the phenethylamine was mixed with 3 mL of Ready Protein (Beckman) scintillation cocktail and counted in a Beckman LS1801. Formate reactions with intact mitochondria were terminated by centrifuging the mitochondria and placing the supernatant fraction at 95 °C in a capped 1.5-mL microfuge tube for 5 min. Formate was determined by enzymatic assay using formate dehydrogenase as described previously (Barlowe & Appling, 1988). Formate production in the sonicated mitochondrial supernatants was terminated by transferring the samples to chilled 1.5-mL microfuge tubes and then incubating them at 95 °C for 5 min. The reaction mixtures were then centrifuged at 10000*g*, and the supernatant was assayed for formate as above.

In some experiments, (6*R*)-[^{14}C]-10-formyl-$H_4PteGlu_1$ was used in place of L-[3-^{14}C]serine as a substrate for CO_2 and formate production in sonicated mitochondrial extracts in the presence or absence of 3.5 mM $NADP^+$ and 8.0 mM ADP. (6*R*)-[^{14}C]-10-formyl-$H_4PteGlu_1$ was prepared enzymatically from $H_4PteGlu_1$ and [^{14}C]formate (Curthoys et al., 1972; Barlowe & Appling, 1988).

Intramitochondrial adenine nucleotides were extracted and measured as described previously (Hampson et al., 1984).

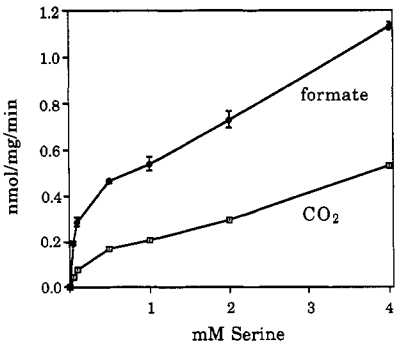

FIGURE 2: Serine dose–response in state-3 mitochondria. Incubation conditions were as described in Experimental Procedures, using 1.0 mM ADP, 30mM glucose, and 7 units of hexokinase as an ADP-regenerating system. Reaction time was 15 min. Each incubation contained 4×10^5 dpm of L-[3-^{14}C]serine. Each point represents the mean ± SEM of duplicate reactions; formate (◆); CO_2 (□).

Pyridine nucleotides (NADH and NADPH) were analyzed using an enzymatic–fluorometric method as described (Williamson & Corkey, 1969).

RESULTS AND DISCUSSION

Previously, we reported initial studies on a mitochondrial pathway for the production of formate from carbon 3 of serine (Barlowe & Appling, 1988). Formate production was shown to depend on mitochondria, and all the enzymes of the proposed pathway were located within the mitochondrial matrix. Here we report further characterization of the mitochondrial component of this pathway using both intact and sonicated mitochondria. As proposed in Figure 1, 10-formyl-H_4folate represents a branch point in mitochondria where the one-carbon unit can be converted to formate or CO_2, among other fates. In fact, Yoshida and Kikuchi (1970, 1971) demonstrated that rat liver mitochondria catalyze the oxidation of carbon 3 of serine to CO_2. In order to better understand this metabolic pathway and its control, we studied its dependence on the metabolic state in intact mitochondria and examined the cofactor requirements in soluble mitochondrial extracts.

Studies on Intact Mitochondria. Intact mitochondria under state-3 conditions (actively respiring), using an ADP regenerating system and serine as the only exogenous oxidizable substrate, were incubated with different concentrations of L-[3-^{14}C]serine. The resulting formate and CO_2 dose–response curves were biphasic (Figure 2). At low serine concentrations (up to 0.1 mM serine), formate was the preferred product with approximately a 4-fold higher rate of production over that for CO_2. At higher serine concentrations (above 1.0 mM), formate production rates were only 2-fold higher than those for CO_2. At low serine concentrations, an active serine transport in mitochondria, as described by Cybulski and Fisher (1977), would support the initial high metabolic rates due to high initial transport rates. At higher serine concentrations, where the serine transporter is presumably saturated, product formation would be limited by slower diffusion of serine into mitochondria. These data may also suggest that different steps become rate limiting as serine concentration changes. At low serine concentrations, the rate-limiting activity may be SHMT (Figure 1, reaction 4), and formate is the final favored product. At higher serine concentrations, as SHMT becomes saturated for serine, a subsequent step might become rate limiting for formate production, perhaps at the 10-formyl-H_4folate branch. As 10-formyl-H_4folate concentrations increase, CO_2 production also increases, ensuring regeneration of the folate coenzyme.

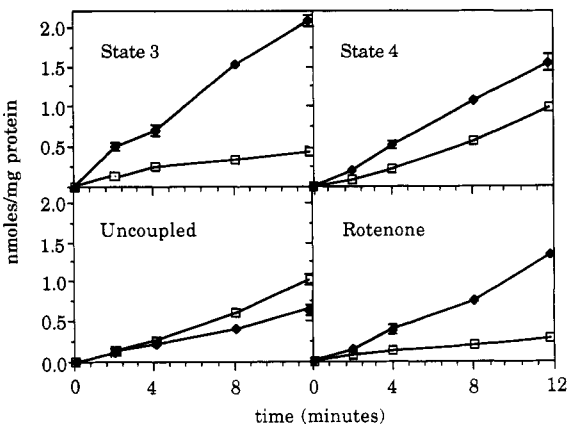

FIGURE 3: The effect of the mitochondrial metabolic state on CO_2 (□) and formate (◆) production from L-[3-^{14}C]serine. Intact mitochondria were maintained in state 3 (using the ADP-regenerating system described in the caption to Figure 2), state 4 (no additions), the uncoupled state (dinitrophenol at a final concentration of 1.0 mM), and a respiratory-inhibited state (rotenone at a final concentration of 2.0 μM). Each point represents the mean ± SEM of duplicate reactions.

Oxidation of carbon 3 of serine to formate and CO_2 was found to be dependent on the metabolic state in which the mitochondria were maintained. The total nanomoles of formate plus CO_2 produced, as well as the formate:CO_2 production ratio, showed slight variation among different batches of mitochondria with different acceptor control ratios (ACRs). ACRs reflect the "intactness" of the mitochondria [a value greater than 4 using succinate as substrate indicates tightly coupled mitochondria (Pedersen et al., 1978)]. Figure 3 shows results from a single batch of mitochondria with an ACR of 5.7. Mitochondria were incubated under a variety of conditions: (a) with an ADP regenerating system (state 3); (b) with no additions other than substrate (state 4); (c) with the uncoupler 2,4-dinitrophenol (DNP); or (d) with the respiratory inhibitor rotenone. L-[3-^{14}C]serine was the only exogenously added oxidizable substrate in all conditions. The rate of formate production was highest in mitochondria under state-3 conditions and lowest in uncoupled mitochondria. CO_2 production was maximal in uncoupled and state-4 mitochondria and lowest when rotenone was present.

All the experiments performed using whole mitochondria relied completely upon the endogenous mitochondrial cofactor pools. As proposed in Figure 1, mitochondrial oxidation of the one-carbon unit derived from carbon 3 of serine to 10-formyl-H_4folate would require one $NADP^+$ (reaction 3). Further metabolism of this one-carbon unit to formate would require one ADP (reaction 1); its oxidation to CO_2 would require a second $NADP^+$ (reaction 7). Adenine nucleotide and NAD(P)H concentrations were determined for mitochondria under each condition (data not shown); the data were in accordance with previously published results (Hampson et al., 1983; 1984). Mitochondria under state-3 conditions showed low concentrations of reduced nicotinamide adenine dinucleotides and high ADP concentrations. The high ADP level and the oxidized $NADP^+$/NADPH pool would favor formate production, as observed (Figure 3). Similarly, CO_2 production was expected to be closely related to the $NADP^+$:NADPH ratio. Indeed, CO_2 production was highest in uncoupled mitochondria, which have the highest $NADP^+$:NADPH ratio, and lowest in rotenone-inhibited mitochondria, which have a low $NADP^+$:NADPH ratio (Figure 3). On the other hand, CO_2 production in state-4 mitochondria was nearly as high as in uncoupled mitochondria, even though state-4

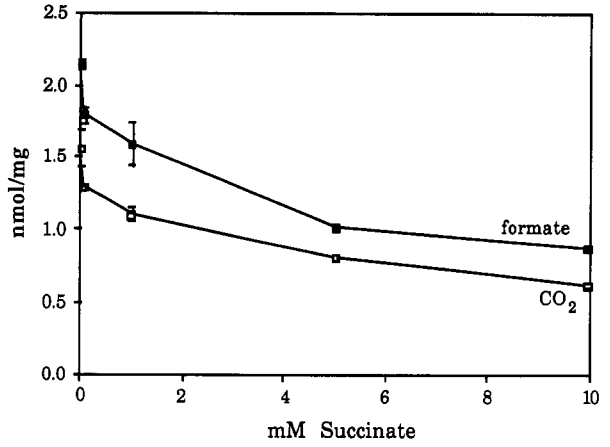

FIGURE 4: Inhibition of serine oxidation by succinate in state-3 mitochondria. CO_2 (□) and formate (◆) production from L-[3-^{14}C]serine was assayed after a 5-min incubation time. Each point represents the mean ± SEM of duplicate reactions.

mitochondria have a very low $NADP^+$:NADPH ratio. Scrutton et al. (1979) showed that the activity of a 10-formyl-H_4folate dehydrogenase (Figure 1, reaction 7), purified from rat liver (most likely cytosolic), was not significantly inhibited in vitro by low $NADP^+$:NADPH ratios. Clearly, there must be other factors in addition to the $NADP^+$:NADPH ratio that contribute to the regulation of CO_2 production.

Addition of a second respiratory substrate (succinate) to mitochondria under state-3 conditions led to a parallel decrease in formate and CO_2 production from L-[3-^{14}C]serine (Figure 4). There was a steep decrease at low succinate concentrations (0.1 to 1.0 mM) and a gentler decrease at higher concentrations of succinate. Fifty percent inhibition was observed at 5 mM succinate (Figure 4). These results are not explained by alterations in the mitochondrial nucleotide pools, as no correlation was found between the decrease in serine oxidation and the NADPH and ADP levels resulting from increasing succinate. Another explanation may be inhibition of serine transport into mitochondria by succinate. If this was the case, then inhibition of serine transport into mitochondria would lead to a parallel decrease in both formate and CO_2 production, independent of the redox state and the adenine nucleotide pools.

Two additional agents known to alter the mitochondrial redox state and adenine nucleotide content are propionate and oligomycin. Propionate has been observed to exert an uncoupling effect in mitochondria (Hampson et al., 1984; Skulachev, 1991) decreasing the concentrations of NADPH and ADP. Oligomycin, on the other hand, increases the ADP levels (Hampson et al., 1984). As expected, state-4 mitochondria and oligomycin-treated mitochondria showed low $NADP^+$:NADPH ratios. Propionate alone and in combination with oligomycin showed high $NADP^+$:NADPH ratios. Also, the ADP content was high if oligomycin was present, all in accordance with previous reports (Hampson et al., 1984). The effects of these compounds on formate and CO_2 production from L-[3-^{14}C]serine in state-4 mitochondria are shown in Table I. The results suggest that formate production is favored when ADP concentrations are high (oligomycin alone or propionate + oligomycin). On the other hand, CO_2 production was highest, and formate production was lowest, when propionate was added. Thus low NADPH and ADP concentrations favor oxidation of the 10-formyl-H_4folate to CO_2.

The partitioning between formate and CO_2 at the 10-formyl-H_4folate branch point probably depends on the cell type, metabolic needs, and species. For example, Yoshida and Kikuchi (1971) demonstrated that, in contrast to rat liver mitochondria, avian liver mitochondria produce very little CO_2 from carbon 3 of serine or carbon 2 of glycine, due to the low activity of 10-formyl-H_4folate dehydrogenase (Figure 1, reaction 7). Instead, avian mitochondria were observed to generate and release an unidentified one-carbon compound which could be utilized for cytoplasmic purine biosynthesis. The authors proposed formate as the one-carbon compound and suggested that, in avian livers, mitochondrial one-carbon units from serine or glycine are oxidized to formate, which leaves the mitochondria for use in purine biosynthesis, whereas in rat liver, the mitochondrial one-carbon units are oxidized mainly to CO_2 (Yoshida & Kikuchi, 1971). Here we show that rat liver mitochondria oxidize serine-derived one-carbon units to both formate and CO_2, regulated partly by the mitochondrial nucleotide content and redox state. We previously demonstrated that, as in the avian system, formate produced by rat liver mitochondria can be incorporated into purines (Barlowe & Appling, 1988). The metabolic role of the 10-formyl-H_4folate dehydrogenase reaction to CO_2 is probably to regenerate intramitochondrial H_4folate (Krebs et al., 1976), whereas the role of the 10-formyl-H_4folate synthetase reaction to formate is to produce a transportable form of one-carbon unit. In a tissue such as rat liver, where the mitochondria can produce either formate or CO_2, the route will depend on the metabolic needs of the cell, mediated in part by changes in mitochondrial nucleotide content and redox state. Other factors, such as allosteric regulation, protein modification, or calcium binding, may also be involved.

Table I: Effect of Oligomycin and Propionate on Serine Oxidation to CO_2 and Formate by Intact Mitochondria[a]

	CO_2 (nmol/mg)	formate (nmol/mg)
state IV (no addition)	1.16 ± 0.10	1.48 ± 0.03
propionate, 5 mM	2.01 ± 0.07	1.06 ± 0.02
oligomycin, 10 μg/mL	1.32 ± 0.02	1.81 ± 0.01
propionate + oligomycin	1.58 ± 0.04	1.98 ± 0.13

[a] Values are means ± SEM at 12 min.

Table II: Cofactor Requirements for CO_2 and Formate Production from Serine by Soluble Mitochondrial Extract[a]

	CO_2 (pmol/min)	formate (pmol/min)
20 μM $H_4PteGlu_5$[b]	196 ● 1	447 ± 3
20 μM $H_4PteGlu_1$[b]	185 ± 4	ND
–folate	ND	ND
–ADP[c]	305 ± 16	ND
–$NADP^+$ [c]	ND	ND

[a] Values are means ±SEM; ND, not detected. [b] Complete system (folate + 300 μM ADP + 350 μM $NADP^+$). [c] 20 μM $H_4PteGlu_5$ as the folate cofactor.

Studies on Sonicated Systems. We next turned to sonicated mitochondria to further characterize the cofactor requirements of L-[3-^{14}C]serine oxidation. As stated above, all of the studies done using intact mitochondria relied entirely on endogenous mitochondrial cofactor pools. Sonication of mitochondria and dialysis of the resulting supernatant fraction effectively abolishes endogenous cofactor pools. This allows the opportunity to test the cofactor requirements proposed in Figure 1. Sonicated mitochondrial supernatant supplied with $NADP^+$, ADP, and the monoglutamate form of the reduced folate cofactor ($H_4PteGlu_1$) was capable of supporting CO_2 production but not formate production (Table II). Addition of NAD^+ or an ADP-regenerating system to drive the 10-formyl-H_4folate synthetase reaction toward formate production had no effect. Formate production by this sonicated mitochondrial supernatant was found to be completely dependent on the

presence of a polyglutamylated reduced folate cofactor (Table II). The pentaglutamate form, $H_4PteGlu_5$, was the only polyglutamate folate tested in these experiments. Consistent with the pathway proposed (Figure 1), CO_2 production was dependent on folate and $NADP^+$, but not on ADP. If preformed 10-[^{14}C]formyl-$H_4PteGlu_1$ is added directly to the sonicated system, we observe a complete dependence on $NADP^+$ for CO_2 production (data not shown), consistent with previous reports suggesting 10-formyl-H_4folate dehydrogenase as the final step for CO_2 production (Scrutton & Beis, 1979; Barlowe & Appling, 1988). Yoshida and Kikuchi (1970, 1971) earlier reported a stimulatory effect of $NADP^+$ and $H_4PteGlu_1$ on CO_2 production from carbon 3 of serine in rat liver mitochondria. When $H_4PteGlu_5$ was used, formate production was dependent on both $NADP^+$ and ADP. For both CO_2 and formate production, the dependance upon added cofactors was greater with extended dialysis of the sonicated supernatant, but the total nanomoles of product decreased as dialysis time increased. These cofactor requirements confirm the pathway proposed in Figure 1. Thus, transfer of carbon 3 of serine to $H_4PteGlu_n$ produces 5,10-methylene-H_4folate; oxidation to 5,10-methenyl-H_4folate requires $NADP^+$; and then, at the 10-formyl-H_4folate branch point, ADP is required for formate production, and $NADP^+$ is required for CO_2 production.

These results do not exclude the possibility of additional, non-folate-dependent pathways, with other cofactor requirements. One alternative route for CO_2 production from carbon 3 of serine is through pyruvate. Serine can be converted to pyruvate by serine dehydratase, and the pyruvate could then be oxidized to CO_2 by the pyruvate dehydrogenase complex and the citric acid cycle. Serine dehydratase is a cytoplasmic enzyme (Yoshida & Kikuchi, 1972), whose main physiological role is the formation of pyruvate from serine for gluconeogenesis (Noda et al., 1990). If oxidation of serine to CO_2 involved pyruvate as an intermediate, addition of excess unlabeled pyruvate should result in a decrease in the $^{14}CO_2$ dpm derived from L-[3-^{14}C]serine. Unlabeled pyruvate was added to a sonicated supernatant system to 1.0 mM (10-fold higher concentration than L-[3-^{14}C]serine), and $^{14}CO_2$ production was monitored. There was no decrease in the $^{14}CO_2$ dpm produced compared to that produced in the absence of pyruvate (data not shown). A second possible pathway involves catalase-mediated CO_2 production from formate (Rapoport et al., 1980). 3-Amino-1,2,4-triazole was added to the sonicated supernatant system to inhibit catalase (Palese & Tephly, 1975). If formate was being further oxidized to CO_2 by catalase, catalase inhibition would lead to a decrease in CO_2 production and a corresponding increase in formate accumulation. However, we observed a parallel decrease in both CO_2 and formate production (data now shown), suggesting a general inhibition of the system. Further characterization of this effect was not studied.

The dependence of CO_2 and formate production on the folate cofactor concentration was examined with a dose–response experiment. A sonicated supernatant system supplemented with $NADP^+$ and ADP was assayed for oxidation of L-[3-^{14}C]serine under varying concentrations of either $H_4PteGlu_1$ or $H_4PteGlu_5$ (Figure 5). Formate production with monoglutamylated H_4folate was barely detectable, even at 90 μM $H_4PteGlu_1$ (data now shown). $H_4PteGlu_5$, however, supported formate production at all concentrations tested. Both forms of the folate cofactor supported CO_2 production, with the pentaglutamate form yielding greater oxidation rates.

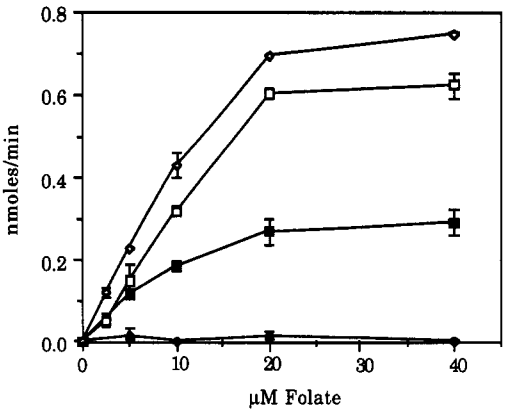

FIGURE 5: Folate cofactor dose–response in sonicated mitochondrial supernatants. Isolated mitochondria were sonicated and dialyzed as described in Experimental Procedures. The supernatants were supplemented with the complete system (Table II) containing either $H_4PteGlu_1$ (closed symbols) or $H_4PteGlu_5$ (open symbols) and assayed for CO_2 (□, ■) and formate (◇, ◆) production from L-[3-^{14}C]serine. $NADP^+$ was present at 350 μM, and ADP, at 300 μM. Each point represents the mean ● SEM of duplicate reactions.

These results are consistent with results from previous studies of the interaction of polyglutamylated folates and the enzymes involved in this pathway (SHMT and C_1-THF synthase). Matthews et al. (1982), studying the cytosolic SHMT from pig liver, showed that the binding energy contributed by the polyglutamate side chain results in lower K_M values for the $H_4PteGlu_n$ substrates, but does not affect the K_M for serine. Strong and Schirch (1989) observed synergistic binding of $H_4PteGlu_4$ and formate with the 10-formyl-H_4folate synthetase activity of rabbit liver cytosolic C_1-THF synthase in the presence of an ATP analogue. More importantly, studies of the interaction of the mitochondrial isoform of SHMT with $H_4PteGlu_n$ (Strong et al., 1989) revealed that reaction in the serine-to-glycine direction was not significantly affected by polyglutamate chain length, but in the glycine-to-serine direction there was a 9-fold increase in k_{cat}/K_m when the longer chain polyglutamates were used. This result was not due to alterations in the K_{eq} by the increased chain length. This observation is reminiscent of our own results. In the crude soluble system reported here, we observe a complete requirement for a polyglutamylated form of the cofactor for formate production, presumably at the 10-formyl-THF synthetase reaction. The monoglutamate form of the cofactor is clearly capable of supporting metabolism to the 10-formyl-H_4folate branch point, as evidenced by CO_2 production (Table II) via 10-formyl-THF dehydrogenase. Thus, while the mitochondrial synthetase is quite active in the direction of 10-formyl-H_4folate synthesis using the monoglutamate form of the folate cofactor (Barlowe & Appling, 1988), formate synthesis requires a polyglutamylated form. The facile production of both CO_2 and formate in intact mitochondria reflects the polyglutamylation state of the endogenous folate pool in mitochondria, since rat liver mitochondria contain predominately the penta- and hexaglutamate forms of the reduced folates (Shin et al., 1976).

The polyglutamate moiety plays a critical, but not completely understood, role in the folate-mediated enzymatic processes. Presence of the multiple negative charges of the polyglutamate tail on the folate cofactor make it more difficult for the molecule to cross membranes. Indeed, a role for the polyglutamate tail in the retention of cellular folates has been proposed (McBurney & Whitmore, 1974; Taylor & Hanna, 1977). In addition, as mentioned above, many enzymes exhibit differential affinities for the polyglutamylated forms of the

folate cofactors (Coward et al., 1975; Matthews et al., 1982; Strong & Schirch, 1989). A classification of the folate-utilizing enzymes with respect to their dependence on the polyglutamate portion of the coenzyme has been proposed (Green et al., 1988). It could be argued that cellular retention may have been the initial role for the polyglutamylated moiety and that the folate-utilizing enzymes have evolved different affinities for the polyglutamylated chains, depending on their metabolic role.

In the in vitro experiments presented here, the mitochondrial pathway of one-carbon interconversions was studied unidirectionally, i.e., from serine to formate and CO_2. Recently, we observed the operation of this pathway in yeast using ^{13}C-NMR (Pasternack et al., 1992). That study provided clear evidence of mitochondrial assimilation of formate into serine and glycine in vivo. Thus, it is important to keep in mind that the pathway is reversible, and understanding how the flux is controlled in vivo represents the next challenge. Considering the argument raised by Srere (1987) that approximately 80% of the major metabolic intermediates have just one use in the cell, it is necessary to call for a very high level of organization, including channeling and compartmentation, to account for the efficiency of cellular metabolism. Here we report the initial characterization of a compartmentalized pathway for folate-mediated one-carbon units. Further studies of this system, including characterization of its sensitivity to second messengers, purification of the enzymes involved, and use of existing mutant cells to characterize the involvement of the mitochondria in the production and usage of one-carbon units, is needed.

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REFERENCES

Appling, D. R. (1991) *FASEB J. 5*, 2645–2651.
Bailey, J. L. (1967) *Techniques in Protein Chemistry*, 2nd ed., pp 340–341, Elsevier, Amsterdam.
Barlowe, C. K., & Appling, D. R. (1988) *Biofactors 1*, 171–176.
Barlowe, C. K., Williams, M. E., Rabinowitz, J. C., & Appling, D. R. (1989) *Biochemistry 28*, 2099–2106.
Blakley, R. L. (1957) *Biochem. J. 65*, 331–342.
Coward, J. K., Chello, P. L., Cashmore, A. R., Parameswaran, K. N., DeAngelis, L. M., & Bertino, J. R. (1975) *Biochemistry 14*, 1548–1552.
Curthoys, N. P., & Rabinowitz, J. C. (1971) *J. Biol. Chem. 246*, 6942–6952.
Curthoys, N. P., Scott, J. M., & Rabinowitz, J. C. (1972) *J. Biol. Chem. 247*, 1959–1964.
Cybulski, R. L., & Fisher, R. R. (1977) *Biochemistry 16*, 5116–5120.
Green, J. M., MacKenzie, R. E., & Matthews, R. G. (1988) *Biochemistry 27*, 8014–8022.
Hampson, R., Barron, L., & Olson, M. (1983) *J. Biol. Chem. 258*, 2993–2999.
Hampson, R. K., Barron, L. L., & Olson, M. S. (1984) *Biochemistry 23*, 4604–4610.
Krebs, H. A., Hems, R., & Tyler, B. (1976) *Biochem. J. 158*, 341–353.
Matthews, R. G., Ross, J., Baugh, C. M., Cook, J., & Davis, L. (1982) *Biochemistry 21*, 1230–1238.
McBurney, M. W., & Whitmore, G. F. (1974) *Cell 2*, 173–182.
Noda, C., Ohguri, M., & Ichihara, A. (1990) *Biochem. Biophys. Res. Commun. 168*, 335–342.
Palese, M., & Tephly, T. R. (1975) *J. Toxicol. Environ. Health 1*, 13–24.
Pasternack, L. B., Laude, D. A., Jr., & Appling, D. R. (1992) *Biochemistry 31*, 8713–8719.
Pedersen, P. L., Greenawalt, J. W., Reynafarje, B., Hullihen, J., Decker, G. L., Soper, J. W., & Bustamente, E. (1978) *Methods Cell Biol. 20*, 411–481.
Rabinowitz, J. C. (1985) in *Methods of Enzymatic Analysis* (Bergmeyer, J., Ed.) 3rd ed., Vol. VII, pp 487–493, VCH, Weinheim.
Rapoport, S., Muller, M., Dumdey, R., & Rathmann, J. (1980) *Eur. J. Biochem. 108*, 449–455.
Rickwood, D., Wilson, M. T., & Darley-Usmar, V. M. (1987) in *Mitochondria. A Practical Approach* (Darley-Usmar, V. M., Rickwood, D., & Wilson, M. T., Eds.) pp 1–16, IRL Press, Washington, DC.
Schirch, L. (1984) in *Folates and Pterins* (Blakley, R. L., & Benkovic, S. J., Eds.) Vol. 1, pp 399–431, Wiley, New York.
Schirch, V., Hopkins, S., Villar, E., & Angelaccio, S. (1985) *J. Bacteriol. 163*, 1–7.
Scrutton, M. C., & Beis, I. (1979) *Biochem. J. 177*, 833–846.
Shin, Y. S., Chan, C., Vidal, A. J., Brody, T., & Stokstad, E. L. R. (1976) *Biochim. Biophys. Acta 444*, 794–801.
Skulachev, V. P. (1991) *FEBS Lett. 294*, 158–162.
Srere, P. A. (1987) *Annu. Rev. Biochem. 56*, 89–124.
Stover, P., & Schirch, V. (1992) *Anal. Biochem. 202*, 82–88.
Strong, W. B., & Schirch, V. (1989) *Biochemistry 28*, 9430–9439.
Strong, W. B., Cook, R., & Schirch, V. (1989) *Biochemistry 28*, 106–114.
Taylor, R. T., & Hanna, M. L. (1977) *Arch. Biochem. Biophys. 181*, 331–344.
Williamson, J. R., & Corkey, B. E. (1969) *Methods Enzymol. 13*, 434–509.
Yoshida, T., & Kikuchi, G. (1970) *Arch. Biochem. Biophys. 139*, 380–392.
Yoshida, T., & Kikuchi, G. (1971) *Arch. Biochem. Biophys. 145*, 658–668.
Yoshida, T., & Kikuchi, G. (1972) *J. Biochem. 72*, 1503–1516.