Oxidation of 10-Formyltetrahydrofolate to 10-Formyldihydrofolate by Complex IV of Rat Mitochondria

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ABSTRACT: We hypothesized that the unanticipated bioactivity of orally administered unnatural carbon-6 isomers, (6*R*)-5-formyltetrahydrofolate (5-HCO-THF) and (6*S*)-5,10-methenyltetrahydrofolate (5,10-CH-THF), in humans [Baggott, J. E., and Tamura, T. (1999) *Biochim. Biophys. Acta 1472*, 323−32] is explained by the rapid oxidation of (6*S*)-10-formyltetrahydrofolate (10-HCO-THF), which is produced by in vivo chemical processes from the above folates. An oxidation of 10-HCO-THF produces 10-formyldihydrofolate (10-HCO-DHF), which no longer has the asymmetric center at carbon-6 and is metabolized by aminoimidazole carboxamide ribotide (AICAR) transformylase forming bioactive dihydrofolate. Since cytochrome *c* (Fe^{3+}) rapidly oxidizes both (6*R*)- and (6*S*)-10-HCO-THF [Baggott et al. (2001) *Biochem. J. 354*, 115−22], we investigated the metabolism of 10-HCO-THF by isolated rat liver mitochondria. We found that 10-HCO-THF supported the respiration of mitochondria without uncoupling ATP synthesis. The site of electron donation was identified as complex IV, which contains cytochrome *c*; the folate product was 10-HCO-DHF, and the reaction was saturable with respect to 10-HCO-THF. Both (6*S*)-(unnatural) and (6*R*)-10-HCO-THF supported the respiration of mitochondria, whereas (6*S*)-5-formyltetrahydrofolate (5-HCO-THF) was inactive. To our knowledge, this cytochrome *c* oxidation of 10-HCO-THF to 10-HCO-DHF in the mitochondrial intermembrane space represents a possible folate metabolic pathway previously unidentified and would explain the bioactivity of unnatural carbon-6 isomers, (6*R*)-5-HCO-THF and (6*S*)-5,10-CH-THF, in humans.

Several lines of evidence in the literature suggest the rapid in vivo oxidation of 10-formyltetrahydrofolate (10-HCO-THF)[1] to 10-formyldihydrofolate (10-HCO-DHF) (*1*−*4*). These include the rapid formation of radiolabeled 10-formylfolic acid (10-HCO-F) from radiolabeled folic acid (pteroylglutamic acid) and the unexpected bioactivity of oral doses of the unnatural carbon-6 isomers (6*R*)-5-formyltetrahydrofolate (5-HCO-THF) and (6*S*)-5,10-methenyltetrahydrofolate (5,10-CH-THF) in humans (*1*, *2*). 5-HCO-THF and 5,10-CH-THF are converted by chemical processes to 10-HCO-THF (*2*). The oxidation of 10-HCO-THF to 10-HCO-DHF and subsequent oxidation to 10-HCO-F explain the formation of 10-HCO-F. The first oxidation removes the asymmetric center at carbon-6, and the resulting 10-HCO-DHF is utilized by aminoimmidazole carboxamide ribotide transformylase (AICAR transformylase) to form dihydrofolate (DHF) and inosine monophosphate (*3*). Since DHF is metabolically active, this process explains the bioactivity of the above unnatural carbon-6 isomers. Recently, Baggott et al. (*4*) reported that (6*RS*)-10-HCO-THF is converted to 10-HCO-DHF by a rapid nonenzymatic reaction with oxidized cytochrome *c*, thereby reducing the cytochrome. These findings lead us to hypothesize that the oxidation of 10-HCO-THF to 10-HCO-DHF takes place in mitochondria, that 10-HCO-THF supports mitochondrial respiration, and that cytochrome *c* is an oxidant.

MATERIALS AND METHODS

(6*S*)-5-HCO-THF was a gift from Lederle Laboratories (Pearl River, NY). The racemic mixture (6*RS*)-5-HCO-THF (Sigma Chemical, St. Louis, MO) was used to prepare (6*RS*)-5,10-CH-THF, and the concentrations of both 10-HCO-THF and 5,10-CH-THF were estimated as previously described (*4*). No antioxidants were used in the experiments described here; thus, it created a problem in terms of using the labile 10-HCO-THF. The formation of 10-HCO-THF from 5,10-CH-THF in the mitochondrial respiration buffer was allowed to proceed to 50% of complete conversion at room temperature and represented a compromise between complete conversion and the prevention of excessive oxidation of 10-HCO-THF. After 50% conversion, the solution was placed on ice until used. This solution contained 25% (6*R*)- and 25% (6*S*)-5,10-CH-THF and 25% (6*R*)- and 25% (6*S*)-10-HCO-THF. Thus, concentrations of 10-HCO-THF are given (Figures 1−3) with the understanding that an equal concen-

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[1] Abbreviations: 10-HCO-THF, 10-formyltetrahydrofolate; 10-HCO-DHF, 10-formyldihydrofolate; 5-HCO-THF, 5-formyltetrahydrofolate; 5,10-CH-THF, 5,10-methenyltetrahydrofolate; 10-HCO-F, 10-formylfolic acid; THF, tetrahydrofolate; DHF, dihydrofolate; AICAR, aminoimidazole carboxamide ribotide; mitos, mitochondria.

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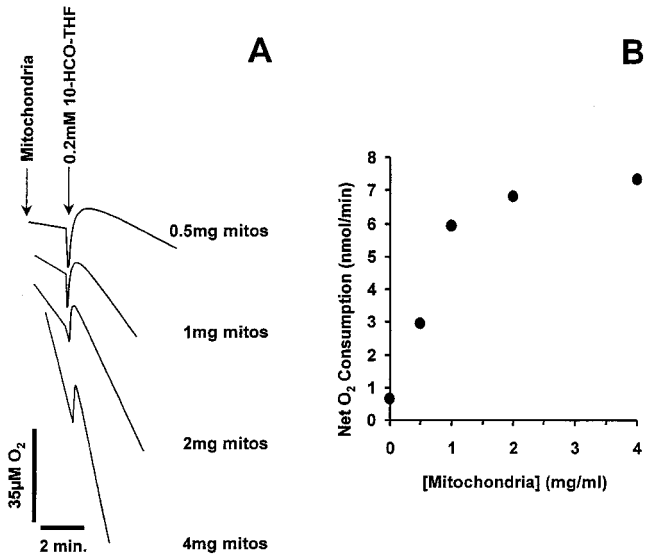

FIGURE 1: Dependence of 10-HCO-THF-supported respiration on the concentration of mitochondria. (A) Mitochondria were incubated in the chamber at the protein concentrations shown. At the arrow, 10-HCO-THF (0.2 mM final concentration) was added, and respiration followed. Representative O_2 traces are shown. (B) Net O_2 consumption rate (O_2 consumption rate with 10-HCO-THF minus O_2 consumption rate without 10-HCO-THF). Rates are expressed as nmol/min, but are not normalized to protein, since different protein concentrations are used in each incubation.

tration of 5,10-CH-THF was also present. The (6*S*)-isomers of these folates are not naturally occurring.

Eight-week-old, male Sprague−Dawley rats (Harlan, Indianapolis, IN) were given food and water ad libitum in a room with 12 h dark/light cycle. Rat liver mitochondria were prepared by differential centrifugation in buffer (pH 7.2) containing 250 mM sucrose, 10 mM Tris, 1.0 mM EGTA as previously described (*5*). Mitochondrial protein concentration was determined as described by Lowry et al. (*6*). The cytochrome *c* content of the rat liver mitochondria is known to be about 0.24 nmol/mg of protein (*7*).

Respiration experiments were performed using a Clark-type oxygen electrode (Instech, Plymouth Meeting, PA) in a custom-made 1.0 mL Perspex chamber at 37 °C. The respiration buffer (pH 7.2) consisted of 120 mM KCl, 25 mM sucrose, 10 mM Hepes, 1.0 mM EGTA, 5.0 mM potassium phosphate, and 1.0 mM $MgCl_2$.

Oxidation of 10-HCO-THF was carried out at 23 °C in 2.5 mL of respiration buffer containing 10 mg of mitochondrial protein in a 15 mL tube. The oxidation of 10-HCO-THF to 10-HCO-DHF was monitored as previously described (*8*). The tubes were gently inverted at the beginning of the reaction and before an aliquot was removed at each time point (Figure 4). In a control experiment, mitochondria were not added to the reaction.

RESULTS

Figure 1 shows that the 10-HCO-THF-driven O_2 consumption depended on the amount of mitochondria added to the respiration chamber with a constant concentration of 10-HCO-THF. The O_2 consumption reached a plateau at 2.0 mg/mL of mitochondria protein possibly because the rate of O_2 consumption became equal to the rate of hydrolysis of 5,10-CH-THF to 10-HCO-THF. Figure 1 also shows an endogenous rate of oxidation (i.e., before the addition of 10-HCO-THF) probably due to endogenous substrates and those released by mitochondrial death. As expected, this endogenous rate increased with the amount of mitochondria added. These substrates may effectively compete with 10-HCO-THF, also resulting in a plateau of O_2 consumption with increasing mitochondrial concentration.

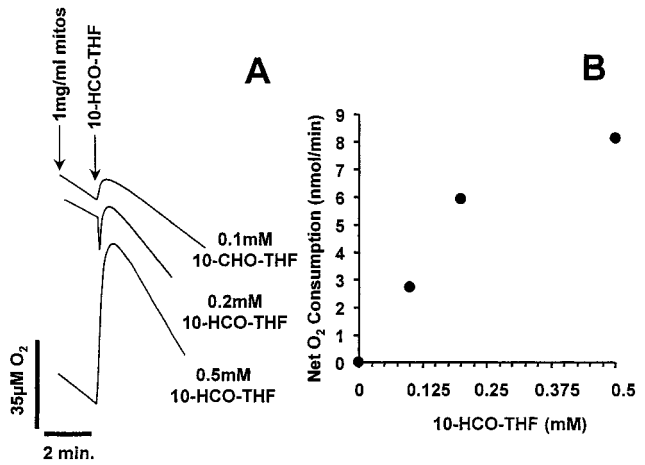

FIGURE 2: Dependence of 10-HCO-THF-supported mitochondrial respiration on the concentration of 10-HCO-THF. (A) Mitochondria were incubated in the chamber at 1.0 mg of protein/mL. At the arrow, 10-HCO-THF was added, yielding the indicated final concentrations. Representative O_2 traces are shown. (B) Net O_2 consumption rate (O_2 consumption rate with 10-HCO-THF minus O_2 consumption rate without 10-HCO-THF).

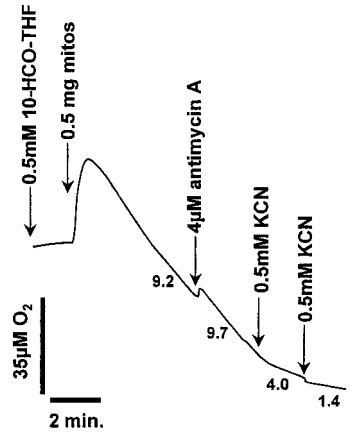

FIGURE 3: 10-HCO-THF-supported mitochondrial respiration is inhibited by KCN but not by antimycin A. 10-HCO-THF was incubated in the chamber at 1.0 mM, followed by the addition of mitochondria (0.5 mg of protein/mL) as shown by the arrow. Further additions were made as indicated by subsequent arrows. Numbers adjacent to the traces indicate O_2 consumption rates in nmol/min.

Figure 2 depicts the dependence of O_2 consumption on the concentrations of 10-HCO-THF at a constant amount of mitochondria. As shown in Figures 1 and 2, there was a transient increase in O_2 concentration with addition of 10-HCO-THF. This was probably caused by adding 10-HCO-THF in a solution stored at 0 °C which contained a higher O_2 concentration than the solution at 37 °C. The rate of O_2 consumption appeared to be saturable with increasing 10-HCO-THF concentration.

The mitochondria respiration driven by 10-HCO-THF was not inhibited by antimycin A, whereas it was inhibited by KCN (Figure 3). The respiration driven by 10-HCO-THF occurred in both states IV (Figure 3) and III with the addition of ADP (data not shown). Thus, 10-HCO-THF did not cause

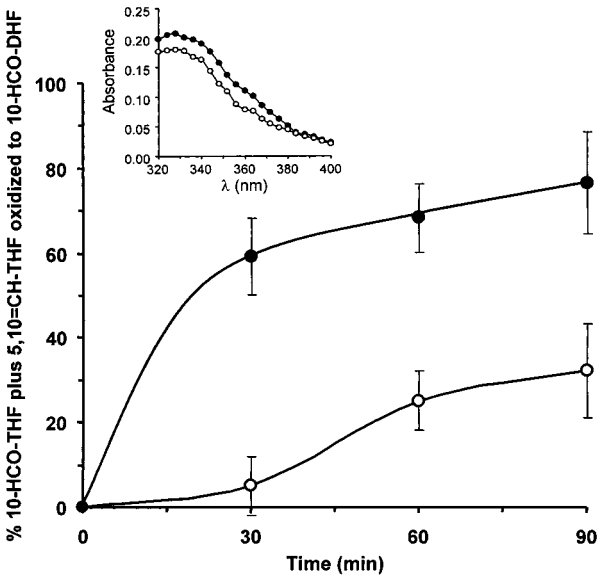

FIGURE 4: Oxidation of 10-HCO-THF to 10-HCO-DHF by mitochondria at 23 °C. 10-HCO-THF [2.5 mL of a solution of equal concentrations (400 μM) of 10-HCO-THF and 5,10-CH-THF] was incubated with mitochondria (10 mg of protein) in the respiration buffer (closed circles). At the times indicated, a 0.1 mL aliquot was removed and diluted with 1.0 mL of 0.1 M H_2SO_4, and the A_{356} was read after 20 min. The A_{356} was converted to percent of 10-HCO-THF plus 5,10-CH-THF oxidized to 10-HCO-DHF (*8*). A control experiment without mitochondria was also performed (open circles). All points are means (±SD) of three separate experiments. The inset shows the UV spectra of the folate products in 0.1 M H_2SO_4 after a 16 h reaction.

an uncoupling of mitochondrial ATP synthesis. The chemically similar folate, 5-HCO-THF, is known to be stable to oxidation, and it did not support mitochondrial respiration (data not shown).

Figure 4 shows the formation of 10-HCO-DHF from 10-HCO-THF in the presence and absence of mitochondria. In a 30 min period, 59% of total 10-HCO-THF plus 5,10-CH-THF which had been exposed to mitochondria was oxidized to 10-HCO-DHF, whereas only 6% was oxidized in the experiment without mitochondria. At 90 min, 76% of the total folate incubated with mitochondria was oxidized to 10-HCO-DHF. The $t_{1/2}$ for the hydrolysis of 5,10-CH-THF to 10-HCO-THF in the respiration buffer was 87 min. The inset in Figure 4 shows the UV spectrum (in 0.1 M H_2SO_4) of the folate product after exposure of 10-HCO-THF to mitochondria for 16 h. The same spectrum was obtained after 16 h in the control reaction. Both spectra were similar to the published UV spectrum of 10-HCO-DHF in acid (*3*, *8*).

DISCUSSION

Our data indicate that 10-HCO-THF drove mitochondrial respiration (Figures 1 and 2). The data in Figure 3 identify the site of electron donation as complex IV, with cytochrome *c* (Fe^{3+}) as the electron acceptor, since respiration was not inhibited by antimycin A but was inhibited by KCN. Since it is known that two cytochromes *c* (Fe^{3+}) oxidize one 10-HCO-THF (*4*) and that $^1/_2O_2$ is required to reoxidize two cytochromes *c* (Fe^{2+}) (*9*, *10*), the following stoichiometry is obtained:

$$\text{10-HCO-THF} + {}^1/_2O_2 \rightarrow \text{10-HCO-DHF} + H_2O$$

To our knowledge, this is the first experimental evidence indicating that 10-HCO-THF was oxidized to 10-HCO-DHF in isolated rat mitochondria in vitro. The findings indicate that this mitochondrial oxidation may take place in vivo with a possibly significant physiological and metabolic role.

The oxidation of 10-HCO-THF by mitochondria must be nonspecific for the carbon-6 optical isomer, since within 30 min, virtually all (6*RS*)-10-HCO-THF, that was initially present and produced from the hydrolysis of (6*RS*)-5,10-CH-THF (i.e., 59% of the total folate), was oxidized (Figure 4). Thus, there appears to be no preference for one isomer. This is consistent with the lack of preference in the chemical reactions of (6*RS*)-10-HCO-THF with cytochrome *c* previously reported (*4*). After this initial burst of the formation of 10-HCO-DHF, it is likely that hydrolysis of 5,10-CH-THF to 10-HCO-THF became rate-limiting. The following calculation from data in Figure 4 indicates that cytochrome *c* must have been reduced and reoxidized. During the first 30 min, 53% (i.e., 59 − 6 = 53) of 2.5 mL of a 400 μM solution of 10-HCO-THF plus 5,10-CH-THF was oxidized to 10-HCO-DHF by mitochondria. This indicates that 53% of 1.0 μmol (i.e., 4.0×10^{-4} M $\times$ 2.5×10^{-3} L) or 530 nmol of 10-HCO-DHF was produced. Based on the previously published stoichiometry that two cytochromes must be reduced for each 10-HCO-THF oxidized to 10-HCO-DHF (*4*), the 530 nmol of 10-HCO-DHF would require 1,060 nmol of cytochrome *c*. Since 10 mg of mitochondrial protein containing only 2.4 nmol of cytochrome *c* was added, the cytochrome *c* present was recycled 440 times (i.e., 1060 ÷ 2.4 = 440).

The data presented here suggest that mitochondrial cytochrome *c* oxidation of (6*S*)-10-HCO-THF to 10-HCO-DHF is the explanation for the unexpected bioactivity of precursors of this unnatural isomer in humans (*2*). 10-HCO-DHF is metabolically active and can be utilized by AICAR transformylase, the last folate-dependent reaction in purine nucleotide biosynthesis (*3*). The data also indicate that mitochondrial oxidation of the natural isomer, (6*R*)-10-HCO-THF, indeed takes place. The mitochondrial oxidation of 10-HCO-THF may account for the rapid appearance of 10-HCO-DHF and 10-HCO-F following doses of folic acid in humans (*1*).

Why do mitochondria oxidize 10-HCO-THF to 10-HCO-DHF? Several nonmutually exclusive reasons are plausible. First, since 10-HCO-DHF is preferred over 10-HCO-THF by mammalian AICAR transformylase (*3*), it is possible that purine nucleotide biosynthesis is regulated by the mitochondrial respiration rate (i.e., cytochrome *c* recycling). As shown in Figure 5, 10-HCO-THF (and presumably its polyglutamates) could diffuse through the outer mitochondrial membrane pore or voltage-dependent anion channel, which is permeable to molecules less than 3000−5000 daltons (*9*−*11*). It is oxidized to 10-HCO-DHF by cytochrome *c* (Fe^{3+}), a soluble and mobile electron carrier located in the intermembrane space (*9*, *10*). Cytochrome *c* (Fe^{2+}) is then reoxidized by complex IV which consumes O_2. After diffusing out of the intermembrane space, 10-HCO-DHF is utilized by AICAR transformylase (*3*), and the resulting DHF is reduced back to THF catalyzed by DHF reductase. Finally, there are several cytosolic metabolic pathways for converting THF back to 10-HCO-THF in order to complete this cycle. Thus, purines may be formed more rapidly by utilizing a

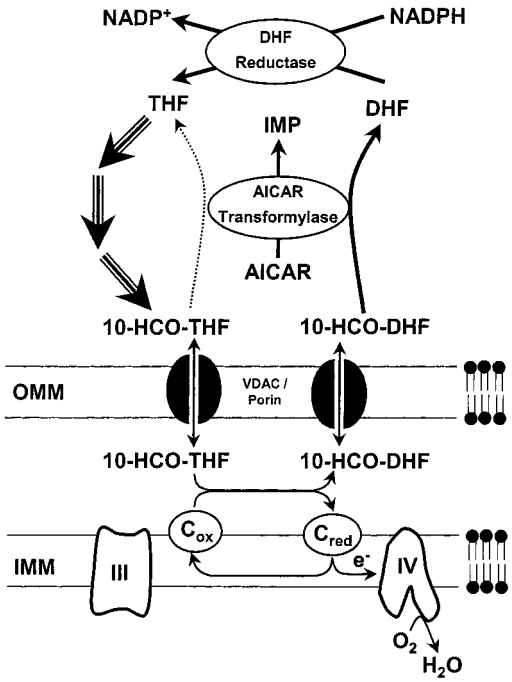

FIGURE 5: Proposed scheme of 10-HCO-THF oxidation by mitochondria. 10-HCO-THF diffuses through the outer mitochondrial membrane (OMM) through the voltage-dependent anion channel (VDAC) or pore (porin) and is oxidized by cytochrome *c* (C_{ox}) to 10-HCO-DHF in the space between the OMM and the inner mitochondrial membrane (IMM). The reduced cytochrome *c* (C_{red}) is reoxidized by complex IV, resulting in O_2 consumption, while 10-HCO-DHF can diffuse through the VDAC/porin to cytoplasm. 10-HCO-DHF is preferentially utilized by AICAR transformylase (solid line) over 10-HCO-THF (broken line). DHF can be reduced to THF catalyzed by DHF reductase and multiple pathways (arrows with triple lines) which metabolize THF back to 10-HCO-THF completing the cycle.

preferred substrate, 10-HCO-DHF, in concert with mitochondria which are more rapidly respiring (Figure 5). Since 10-HCO-THF reacts with cytochrome *c* in the intermembrane space, there is no requirement to transport it into the matrix. Folates stable to cytochrome *c* oxidation, such as 5-HCO-THF and 5,10-CH-THF (*4*), could be transported unaltered into the mitochondrial matrix.

Second, 10-HCO-THF could be an alternate electron transport molecule like $FADH_2$. Once AICAR transformylase removes the formyl group, the resulting DHF can be reduced to THF catalyzed by DHF reductase utilizing NADPH (Figure 5). Thus, THF is free to acquire another formyl group producing 10-HCO-THF. NADPH, through this folate metabolism, could donate electrons to mitochondria.

Third, 10-HCO-THF may protect the cytoplasm from nonproductive or damaging cytochrome *c* oxidation. Only oxidized cytochrome *c*, which has escaped to the cytoplasm, induces apoptosis (*12*). The reduction of cytoplasmic cytochrome *c* by adequate 10-HCO-THF may protect the cell from cytochrome *c*-induced apoptosis.

The reactions catalyzed by thymidylate synthase and the mitochondrial oxidation of 10-HCO-THF are the only known pathways for the in vivo formation of 7,8-dihydrofolates from tetrahydrofolates. The former reaction is quintessential in pyrimidine biosynthesis, and the latter may supply a one-carbon fragment for purine biosynthesis (*3*). In each case, the DHF produced must be reduced back to THF, catalyzed by DHF reductase, in order for folate-dependent one-carbon metabolism to continue. The importance of this reductase may now be even greater than previously thought, since its activity could potentially exert concerted regulation of the synthesis of both thymidylate and purines. Finally, 10-HCO-THF is a labile molecule, and mitochondrial/cytochrome *c* may not be the only in vivo site/mechanism for its oxidation.

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