Published in final edited form as:
Arch Biochem Biophys. 2006 November 15; 455(2): 175–187.

Methenyltetrahydrofolate Synthetase is a High-Affinity Catecholamine-Binding Protein[1]

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Abstract

Recombinant mouse 5,10-methenyltetrahydrofolate synthetase (MTHFS) was expressed in *Escherichia coli* and shown to co-purify with a chromophore that had a λ_{max} at 320 nm. The chromophore remained bound to MTHFS during extensive dialysis, but dissociated from MTHFS when its substrate, 5-formyltetrahydrofolate, was bound. The chromophore was identified as an oxidized catecholamine by mass spectrometry and absorption spectroscopy. Purified recombinant mouse MTHFS and rabbit liver MTHFS proteins were shown to bind oxidized *N*-acetyldopamine (NADA) tightly. The addition of NADA to cell culture medium accelerated markedly folate turnover and decreased both folate accumulation and total cellular folate concentrations in MCF-7 cells. Expression of the MTHFS cDNA in MCF-7 cells increased the concentration of NADA required to deplete cellular folate. The results of this study are the first to identify a link between catecholamines and one-carbon metabolism and demonstrate that NADA accelerates folate turnover and impairs cellular folate accumulation in MCF-7 cells.

Keywords

5; 10-methenyltetrahydrofolate synthetase; MTHFS; catecholamines; folate; 5-formyltetrahydrofolate; *N*-acetyldopamine; one-carbon metabolism; NADA

ABBREVIATIONS

MTHFS - 5,10 - methenyltetrahydrofolate synthetase; DHF - dihydrofolate; THF - tetrahydrofolate; NADA - N-acetyldopamine; oxNADA - oxidized N-acetyldopamine; AdoMet - S-adenosylmethionine; pABG - para-aminobenzoyl(poly)glutamate; ESI-MS - electrospray ionization mass spectrometry; MALDI-TOF - matrix-assisted laser desorption ionization, time-of-flight; ICP-MS - inductively coupled plasma mass spectrometry; MS - mass spectrometry; *E. coli* - Escherichia coli; αMEM - α-minimal essential medium

The term “folate” refers to a family of metabolic cofactors that chemically activate and carry one-carbon units at three different oxidation states. The one-carbon groups carried by tetrahydrofolate (THF) are required for the *de novo* synthesis of purines (supplying the carbon-2 and carbon-8 of the purine ring) and thymidylate (for the methylation of deoxyuridine 5′-monophosphate), and for the remethylation of homocysteine to methionine [1,2]. *S*-adenosylmethionine (AdoMet), formed by the adenylation of methionine, is a cofactor and one-carbon donor for numerous cellular methylation reactions and is a metabolic intermediate in polyamine biosynthesis [3–5]. Folate-dependent pathways are essential for both DNA

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synthesis and cellular methylation reactions. Inadequate intracellular folate accumulation affects both genome stability and gene expression, and increases the risk for certain cancers and developmental anomalies [6].

The regulation of intracellular folate concentrations is influenced by uptake, polyglutamylation, export, turnover, and catabolism [7,8]. Folate monoglutamates are transported into the cell either by folate receptor proteins or reduced folate carrier proteins [9,10]. Intracellular folate monoglutamates are converted to folate polyglutamates by the enzyme folylpolyglutamate synthetase. The polyglutamate tail increases the affinity of the cofactor for folate-binding proteins and is necessary to sequester folates inside the cell and within intracellular compartments [11–14]. Both folate transport and polyglutamylation are essential for cellular folate uptake and retention, but there is no evidence that either of these processes regulates cellular folate concentrations [8,15]. Cellular folate can be depleted by oxidative degradation of labile folate cofactors, otherwise known as folate catabolism. Folate molecules are comprised of a 2-amino-4-hydroxypteridine group linked to *para*-aminobenzoyl (poly)glutamate (*p*ABG) though a methylene carbon bridge [16]. Folate oxidation can occur by two distinct chemical mechanisms [17]. Oxidation of the tetrahydropteridine ring results in the formation of dihydro forms of the folate cofactor (DHF, 5-methylDHF), which can be enzymatically reduced back to the active tetrahydro forms. Alternatively, folate oxidation can result in irreversible degradation of the folate molecule when bond cleavage between C^9 and N^{10} occurs, generating a pterin and *p*ABG species (Figure 1) [7]. The reduced folate derivatives differ in their susceptibility to undergo oxidative degradation [17,18]. 10-formylTHF and the unsubstituted forms of folate (THF and dihydrofolate (DHF)) are chemically labile and are rapidly oxidized *in vitro* [17,18]. It is assumed that unbound intracellular folates are susceptible to oxidative degradation *in vivo* because of the instability of the reduced folate derivatives *in vitro* [7].

Folate catabolism (Figure 1) is thought to be the predominant mechanism of folate turnover in both rat and human models [19] and can deplete intracellular folate concentrations independent of extracellular folate availability and transport [7]. Increased rates of folate catabolism can result in tissue-specific folate deficiency in the absence of whole-body folate deficiency. Rates of folate catabolism are accelerated in cancer models [15] and lead to increased excretion of *p*ABG in human and animal urine [7]. These studies indicate that folate catabolism may be a biologically regulated process. Recently, we have demonstrated that the enzyme methenyltetrahydrofolate synthetase (MTHFS; EC 6.3.3.2) influences intracellular folate concentrations by accelerating rates of folate catabolism [8]. MTHFS catalyzes the ATP-dependent conversion of 5-formylTHF to 5,10-methenylTHF (Reaction 1) [20–22].

5-formylTHF(Glu_n) + MgATP → 5,10-methenylTHF(Glu_n) + MgADP (Reaction 1) 5-formylTHF does not participate directly in folate-dependent one-carbon transfer reactions, but is an inhibitor of various folate-dependent enzymes [23]. MTHFS is the only enzyme known to utilize 5-formylTHF as a substrate. 5-FormylTHF has been suggested to function as a cellular storage form of folate in non-mammalian systems; it is the most stable reduced folate derivative *in vitro* [24]. Cell lines with increased MTHFS activity display increased rates of folate catabolism and exhibit severe folate deficiency despite the presence of pharmacological levels of folate in culture medium [8]. Two potential mechanisms can account for these observations: (1) MTHFS may accelerate folate catabolism indirectly by altering the distribution of folate cofactors leading to the accumulation of the labile forms of folate, or (2) MTHFS may directly catalyze folate catabolism [8]. For the latter to occur, MTHFS requires a two-electron acceptor molecule to catalyze folate catabolism, however, MTHFS is not known to bind cofactors that facilitate redox chemistry [7]. In order to begin distinguishing between these two mechanisms, the role of MTHFS in catalyzing folate turnover was investigated *in vitro* and in MCF-7 cells.

Materials and Methods

Materials

(6*S*)-[3′,5′,7,9-^{3}H]5-formylTHF (40 Ci/mmol) and (6*S*)-[3′,5′,7,9-^{3}H]-5-methylTHF (30 Ci/mmol) were obtained from Moravek Biochemicals, Inc. (6*R*,6*S*)-5-formylTHF was from SAPEC, and (6*S*)-5-methylTHF and (6*S*)-5-formylTHF were the generous gift of Eprova AG. HEPES, ATP, Tris, sodium periodate, dopamine, epinephrine, L-dopa, and *N*-acetyldopamine were purchased from Sigma. Fetal bovine serum, α-minimal essential medium (αMEM), and α-modification lacking sodium bicarbonate, folate, ribosides, ribotides, deoxyribosides, and deoxyribotides (defined αMEM) were obtained from Hyclone Laboratories. The human MCF-7 mammary adenocarcinoma cells (ATCC catalog number HTB22) have been described elsewhere [25]. For folate turnover studies, fetal bovine serum was dialyzed against ten volumes of phosphate-buffered saline (PBS) at 4^{o}C for 24 h with buffer changes every 4 h to deplete serum of folate and other small molecules. The serum was then charcoal-treated to remove any remaining folate.

Preparation and Oxidation of NADA

NADA was prepared by dissolving 3 mg of NADA in 1 mL of 20 mM hydrochloric acid, and the acid was neutralized by the addition of an equal volume of 1 M potassium phosphate, pH 7.2. To generate oxidized NADA (oxNADA), 3 mg of sodium periodate (Sigma) was added to the NADA solution followed by an equal volume of 1 M potassium phosphate, pH 7.2 to adjust the pH to 7.0. Oxidized forms of catecholamines are heterogeneous compounds and exist as one or more quinone species that are very reactive and can rapidly undergo nucleophilic addition reactions and oligomerize. Here we define oxidized NADA as oxNADA to reflect this heterogeneity.

Purification of Recombinant Mouse MTHFS

The mouse MTHFS cDNA was isolated from total liver cDNA, cloned into a pET28a expression vector, and expressed in *E.coli* BL21 Star cells (Invitrogen) cultured in LB medium. Recombinant mouse MTHFS protein was purified using metal affinity chromatography as described previously [26].

Removal of the Chromophore from Recombinant Mouse MTHFS Protein

The chromophore was removed from recombinant mouse MTHFS protein either by application of the protein to a 5-formylTHF-sepharose column or by solvent extraction. Recombinant mouse MTHFS protein was applied to a 5-formylTHF-sepharose column equilibrated with 20 mM Tris pH 7.0 and 20 mM 2-mercaptoethanol. The column was washed with water and the chromophore was collected in the flow-through fractions. Alternatively, the chromophore was removed from the protein by extraction with a 1:3 dilution of 100% acetonitrile/water at 95ºC for 15 min. Glass wool was used to separate the precipitated protein from the solvent containing the chromophore. All fractions containing the chromophore were concentrated under vacuum. The dried pellet was resuspended in 100% acetic acid for analysis by ESI-MS. OxNADA standards were prepared in the same buffer as the chromophore and concentrated under vacuum and analyzed by ESI-MS.

Reconstitution of Purified Rabbit Liver MTHFS with NADA

NADA or oxNADA (5 mg) was added to purified rabbit liver MTHFS (0.025 μmoles) and yeast aldehyde dehydrogenase (10 U / sample) and the protein incubated at room temperature for 15 min. The samples were transferred to dialysis tubing (molecular weight cut off = 5000 Da) and dialyzed overnight against 4°C in 2 L of 5 mM potassium phosphate, pH 7.2 with two

buffer changes. Following dialysis, the proteins were clarified by centrifugation, and UV spectrum for each sample was recorded.

Growth of Recombinant Mouse MTHFS in M9 Minimal Medium

BL21 Star cells transformed with the mouse MTHFS cDNA in the pET-28a vector were used to inoculate 5 mL starter cultures of LB containing 50 μg/ml kanamycin. The cultures were grown for 16 h at 37°C, and an aliquot was used to inoculate a 50 ml LB culture with 50 μg/ml kanamycin. The cultures were incubated at 37°C until A_{550nm} = 0.7, the cells were pelleted by centrifugation and used to inoculate 1 L of minimal medium containing 2X M9 salts, 0.2 mM magnesium sulfate, 0.4% glucose, 12.5 mg ferrous iron sulfate, 1X vitamins solution (Gibco), 40 mg of each of the 20 biologically-relevant amino acids (Sigma), and 50 μg/mL kanamycin. The cultures were incubated at 37 °C until A_{550nm} = 0.7, then IPTG (1.5 mM) and 10 mg NADA (dissolved in ethanol/water) were added. The cultures were incubated at 25°C for 5 hours. Cells were harvested by centrifugation and stored at -80ºC.

MALDI-TOF and ESI-MS Experiments

Spectra were obtained with a Bruker Esquire-LC (Bremen, Germany) ion trap mass spectrometer using both positive and negative ion modes. Samples were infused into the source using a Cole-Parmer 74900 series infusion pump at a rate of 1 μL/min through a fused silica line, with methanol or methanol/water (1/1, v/v) as infusion solvents. The drying temperature (heated capillary) was maintained at 225°C using N_2 gas at 4 L/min and nebulizer flow at 16 psi. The HV capillary was 4000 V (−4000 for negative ion spectra), the cap exit offset 78.6 V, with a 55 V difference between Lens 1 and Lens 2, and a 40 V difference between the two skimmers. MS spectra were obtained by averaging 40 spectra from m/z 95-850. Ten to twelve scans were averaged for MS/MS and MSn spectra using a fragmentation rF amplitude between 0.8 and 1.4 and an isolation width of ± 1.5 u. Unit resolution was obtained for all spectra. Data were processed using the Bruker Data Analysis software, and all MS analyses were performed at the BioResource Center of Cornell University.

Determination of Folate Turnover in Cultured Cells

Cell monolayers at 75% confluence were washed with PBS, then labeled for 12 h in defined αMEM lacking folate and glycine and supplemented with 25 nM (6*S*)-[^{3}H]5-formylTHF. For the chase, cell monolayers were washed with 10 mL of PBS, trypsinized, and pelleted by centrifugation. The cells were seeded in triplicate (1 x 10^6 cells) into 100 mm culture plates containing 10 mL of defined αMEM supplemented with 2 μM folic acid. Catecholamines (20 μM dopamine, NADA, and L-dopa) were prepared, then added immediately to the chase medium prior to the addition of labeled cells. Cells were harvested at defined time points, the medium was removed and the tritium in the medium was quantified. The cell monolayers were washed with PBS and lysed with 0.2 M ammonium hydroxide. Tritium remaining in the cells was quantified using a Beckman LS 8100 liquid scintillation counter.

Determination of Radiolabeled Folate Accumulation in Cultured Cells Treated with Catecholamines

Cell monolayers at 70% confluence in 30 mm plates were labeled for 12 h in defined αMEM lacking folate and glycine, but supplemented with 25 nM (6*S*)-[^{3}H]5-formylTHF and 0, 25 μM, 50 μM, 75 μM, 100 μM, or 150 μM NADA, dopamine, or epinephrine. Cells were harvested by washing with 5 mL PBS, trypsinized, and pelleted by centrifugation and viable cells (determined by their ability to exclude trypan blue) were quantified. The cell pellets were lysed with 0.2 M ammonium hydroxide, and the intracellular tritium was quantified using a Beckman LS 8100 liquid scintillation counter.

Microbiological Assay for Total Cellular Folate Concentrations

Cells were cultured to 70% confluence in αMEM, harvested by trypsinization, and cell pellets were stored at -80°C. Cell pellets were divided in two for total protein determinations and total cellular folate analyses. Total cellular folate concentrations were determined using a protocol that has been described previously [17]. Briefly, cells were lysed using an extraction buffer and folate polyglutamates were converted to their corresponding monoglutamate forms using rat conjugase. Cell lysates and folate standards were added to 96-well plates containing *Lactobacillus casei*, and incubated for 18 h at 37°C. Bacterial growth was determined by measuring absorbance at 550 nm and growth values were correlated to folate content using a standard curve that was generated with known folate standards.

Determination of the Binding Affinity of Dopamine and Oxidized Dopamine for MTHFS protein

Purified recombinant mouse MTHFS protein (500 nM) was incubated with various concentrations (100 nM, 250 nM, 500 nM, 1000 nM) of [3,4-[Ring-2,5,6-^{3}H]-dopamine (2500 cpm/μL; Perkin Elmer) and oxidized dopamine for 15 min at 37°C. Oxidized [^{3}H]-dopamine was prepared using tyrosinase and [^{3}H]-dopamine, which were incubated at 37°C for 5 min. A NanoSep 10K spin column was used to separate tyrosinase from the oxidized dopamine product, which was present in the flowthrough. Spin columns were pre-equilibrated with 100 mM MES buffer, pH 6.0. Following addition of the reaction mixture, the samples were centrifuged for 5 min, and the tritium in three 85 μL aliquots of the flowthrough was determined by liquid scintillation. The reactions were preformed in triplicate, and compared to reaction mixtures lacking MTHFS protein.

RESULTS

Identification of a Small Molecule Bound to Recombinant Mouse MTHFS Protein

The murine MTHFS cDNA was cloned in frame with a hexa-histidine tag, expressed in *E.coli* cultured in LB medium, and purified using metal affinity chromatography [26]. The absorption spectrum of the purified, dialyzed recombinant mouse MTHFS protein indicated the presence of an absorption shoulder at 320 nm, indicating that a chromophore was bound to the protein (Figure 2). The UV spectrum of the purified rabbit liver MTHFS protein lacked the shoulder peak at 320 nm (Figure 2), but displayed a less-pronounced absorbance shoulder at 305 nm which was identified by HPLC to be tightly bound 5-formylTHF that did not dissociate during dialysis.

The mass of the recombinant MTHFS protein was determined by MALDI-TOF mass spectrometry (Figure 3). The recombinant mouse MTHFS protein exhibited two peaks with masses of 25221 and 25403 Da, and there were two peaks corresponding to the multiply charged ions (M+2) at m/z = 12613. The theoretical molecular mass of mouse MTHFS with the hexa-histidine tag was calculated to be 25208 Da and analysis by SDS-PAGE yielded a similar value [26]. The peak observed at 25221 Da for the recombinant mouse MTHFS protein is similar to the estimated molecular mass of 25208 Da, whereas the peak at 25403 Da corresponds to the mass of MTHFS plus 182 Da, which represents the presence of the bound chromophore that was observed in the UV spectral studies. MALDI-TOF analysis was repeated three times using recombinant mouse MTHFS protein from different purifications, and the mass differences among the peaks representing halo-MTHFS and apo-MTHFS varied between 179–182 Da. A sample of recombinant mouse MTHFS protein with salts and contaminants removed using C-18 Zip-Tips yielded a mass difference of 190 Da. Examination of the MALDI-TOF spectrum indicated that most of the protein lacked the bound compound (Figure 3, inset).

Identification of the Small Chromophore Molecule as oxidized NADA (oxNADA)

Dialysis of recombinant mouse MTHFS protein was not sufficient to remove the chromophore from the protein. The chromophore was released from the purified recombinant MTHFS protein when 5-formylTHF was included in the dialysis buffer. The mass of the chromophore was not consistent with the presence of either a folate compound or folate degradation product because these molecules have greater masses than that observed for the chromophore. Application of the protein to a 5-formylTHF-sepharose column also released the chomophore from the protein, and the chromophore was identified in the flow-through fraction. The absorption spectrum of the chromophore present in the flow-through fraction is shown in Figure 4A. The flow-through fraction did not contain protein, which was determined by spotting the sample on a nitrocellulose membrane and staining with Coomassie blue (data not shown). The chromophore could also be removed from the protein by extraction with acetonitrile, which precipitated the protein.

The chromophore was removed from the MTHFS protein and identified using electrospray ionization mass spectrometry (ESI-MS). The ESI-MS spectrum of the chromophore (Figure 5) was compared to spectra of various catecholamine standards. Peaks labeled with an asterisk represent peaks that were also observed in mass spectra of NADA samples, and those labeled with crosses differ by one atomic unit from peaks present in the mass spectra of NADA. The molecular mass of NADA is 195 Da, and the positive mass spectrum of the chromophore in Figure 5C contains a peak at m/z of 197 consistent with the presence of this catecholamine. The peak at m/z = 213 in the positive mass spectra of the chromophore (in Figures 5A and 5C) is consistent with a NADA-H_2O compound or 6-hydroxy-NADA (molecular weight = 211 Da), a catecholamine derivative that is formed at low pH [27]. A major peak at m/z = 363 present in the positive mass spectrum of the acetonitrile extracted sample (Table 1) may correspond to a NADA derivative that has dimerized and is also present in the positive mass spectrum for NADA (Table 1). There are also peaks present in the chromophore spectra that were not present in the spectra of NADA, which indicates either that other compounds in addition to NADA co-purify with recombinant mouse MTHFS protein, or that the chromophore is a modified derivative of NADA. Alternatively, some of these peaks could be artifacts arising from the ESI-MS analysis because compounds present in the sample can undergo gas phase chemistry in the instrument, generating a greater number of chemical species (which would not represent true compounds in the original sample). Alternatively, additional compounds may have been generated during the removal of the chromophore from the protein. Evidence for this can be seen in Figure 5; the positive mass spectrum of the chromophore removed using the 5-formylTHF-sepharose column (Figure 5A) is different from the spectrum of the chromophore removed from the protein by acetonitrile extraction (Figure 5C), although predominant peaks at m/z = 175 and m/z = 213 were present in both samples.

The identity of the chromophore molecule as NADA was confirmed using electrospray tandem mass spectroscopy (MS/MS) to determine the collision-induced dissociation patterns for the chromophore and samples of NADA. Three peaks present in the chromophore sample (m/z = 197, 213, 363) were selected for MS/MS fragmentation, and the resulting peaks for each fragmentation are shown in Table 1. The MS/MS fragmentation of the same three mass peaks present in a sample of NADA (m/z = 197, 213, 363) yielded nearly identical fragmentation patterns as the chromophore sample (Table 1), providing strong evidence that the recombinant MTHFS-bound chromophore is a NADA derivative. Furthermore, the absorbance UV spectrum of NADA oxidized with sodium periodate at pH 7.0 (Figure 4B) is identical to the absorption spectrum for the chromophore molecule (Figure 4A).

Reconstitution of MTHFS Proteins with NADA

Bacteria are not known to synthesize catecholamines, indicating that the MTHFS-bound chromophore originated from the culture medium (LB broth). To test this hypothesis, *E.coli* expressing the murine MTHFS cDNA were grown in M9 minimal medium in the presence and absence of NADA. Recombinant mouse MTHFS protein grown in M9 minimal medium without NADA did not contain the chromophore; whereas MTHFS protein expressed in *E.coli* cultured in M9 medium supplemented with 10 mg/L NADA contained a chromophore. This provides strong evidence that MTHFS binds NADA or derivatives thereof. The chromophore was removed from the protein and analyzed by ESI-MS (Figure 6). The spectrum of the MTHFS-bound chromophore (Figure 6B) contains four peaks (m/z = 215, 237, 313, 365) that were not present in the spectrum of the flow-through fractions from protein expressed in M9 medium lacking NADA (Figure 6A). The differences between the ESI-MS spectrum of the chromophore extracted from recombinant MTHFS protein isolated from bacteria cultured in NADA-supplemented minimal medium (Figure 6B) and the ESI-MS spectrum of the chromophore extracted from recombinant MTHFS protein that was isolated from bacteria cultured in a rich broth containing yeast extract (Figure 5) indicate that NADA had likely undergone chemical transformations while in the culture medium or after transport into *E.coli*. Recombinant MTHFS protein that was synthesized in M9 minimal medium supplemented with [^{13}C]-tyrosine, which is a precursor of dopamine and NADA [28], did not contain a bound chromophore, confirming that the enzymes needed to convert tyrosine into dopamine are not present in *E.coli*.

Because the absorption spectrum of purified rabbit liver MTHFS differed from the spectrum of recombinant mouse MTHFS (Figure 2), the affinity of rabbit liver MTHFS for oxNADA was investigated. Catecholamines rapidly oxidize generating highly reactive quinone and semi-quinone compounds that undergo nucleophilic addition reactions leading to the formation of catecholamine oligomers or protein modifications [28]. Therefore, reconstitution studies using oxNADA compounds are hindered by the short half-life of oxidized catecholamines in solution and the ability of these compounds to oxidize and chemically modify proteins (protein side chains can form covalent adducts with oxidized catecholamines) [29,30]. Purified rabbit liver MTHFS, which lacks a shoulder peak at 320 nm, was incubated with NADA, oxNADA, or a phosphate buffer control for 15 min, then the protein samples were dialyzed overnight. Incubation of rabbit liver MTHFS with oxNADA resulted in a change in the absorption spectrum of the protein relative to the control sample, and the absorbance increase occurred in the 300–450 nm range (Figure 7A). The experiment was repeated using recombinant mouse MTHFS protein, and incubation with oxNADA resulted in an increase in the shoulder peak absorbance in the absorption spectrum of the protein (data not shown). The experiment was also repeated using yeast aldehyde dehydrogenase as a negative control, and incubation with NADA and oxNADA did not alter the absorbance spectra of the protein (Figure 7B). Incubation of purified rabbit liver MTHFS with NADA resulted in an increase in the absorbance at 280 nm in the absorption spectrum of the protein indicating that NADA also has affinity for MTHFS (Figure 7A). However, NADA concentrations of 100, 200, 400 μM did not inhibit the MTHFS-catalyzed conversion of 5-formylTHF to 5,10-methenylTHF when MgATP and 5-formylTHF were present at concentrations equal to their K_m (data not shown). Equilibrium binding experiments using [^{3}H]-dopamine and oxidized [^{3}H]-dopamine demonstrated that neither of these compounds could bind to purified rabbit liver or recombinant mouse MTHFS proteins (data not shown, see *Experimental Procedures*), indicating that acetylation is critical for catecholamine binding to MTHFS. In summary, the MTHFS protein can be reconstituted to contain a non-covalently bound oxNADA molecule which does not affect its catalytic activity, and acetylation of dopamine (generating NADA) is necessary for binding of the catecholamine to MTHFS.

Effect of Catecholamines on Folate Turnover, Folate Accumulation, and Total Cellular Folate Concentrations

The effect of exogenous catecholamines and MTHFS on folate turnover rates were determined using pulse-chase analyses in MCF-7 cells. Catecholamines can function as oxidants following oxidation of their diphenolic ring to dopamine-quinone or dopa-quinone species [28,30], so we hypothesized that they may function as electron acceptors for the folate catabolism reaction. Folate turnover in MCF-7 cells labeled with [^{3}H]5-formylTHF is biphasic, with an initial rapid phase of turnover followed by a slower phase [8]. The rapid phase of turnover represents the catabolism of newly imported short chain folate polyglutamates, and the slower phase represents degradation of the longer-chain folate polyglutamates that are likely protein-bound [8,15]. Previous work demonstrated that MTHFS only accelerates the rapid phase of folate turnover, thereby targeting the pool of newly-imported folates for degradation [8]. Addition of 20 μM NADA to the chase medium increased the magnitude of the rapid phase of folate turnover by 12% without affecting the rate of the slow phase of folate turnover in MCF-7 cells (Figure 8A). To determine whether the effects of NADA on folate turnover rates were specific to this catecholamine, the effects of dopamine and L-dopa on folate turnover were assessed by pulse-chase analyses. Inclusion of dopamine in the chase culture medium at 20 μM increased the initial rapid phase of folate turnover by only 4% relative to untreated cells (Figure 8A). The minor effect of dopamine on folate turnover may reflect *N*-acetyltransferase-mediated dopamine acetylation [31,32]. The addition of L-dopa to the chase medium did not affect folate turnover rates compared to untreated MCF-7 cells (Figure 8B). These results demonstrate that either not all catecholamines are capable of increasing folate turnover rates, or that catecholamines differ in their ability to accumulate in MCF-7 cells.

Because the addition of NADA to the chase medium increased folate turnover rates in MCF-7 cells, we investigated the effects of various catecholamines on [^{3}H]folate accumulation (Figure 9) and total cellular folate content (Table 2). For folate accumulation studies, cells were cultured for 12 h in defined αMEM supplemented with 25 nM (6*S*)-[^{3}H]-5-formylTHF and either NADA, dopamine or epinephrine, and the amount of cellular [^{3}H]folate was determined by liquid scintillation (Figure 9). 5-formylTHF was resistant to oxidative degradation by oxNADA and other catecholamines in culture medium (data not shown). Once transported into the cell, [^{3}H]-5-formylTHF equilibrates rapidly into the folate cofactor pool through the catalytic activity of MTHFS [25]. All other reduced forms of folate were readily oxidized by catecholamines *in vitro* (data not shown). Treatment of MCF-7 cells with increasing concentrations of NADA depressed [^{3}H]folate accumulation (Figure 9A) and total cellular folate content in viable cells (Table 2). MCF-7 cells were most sensitive to NADA in the 50 to 75 μM range as determined by three independent outcomes: total cellular folate concentration analyses (Table 2), labeled folate accumulation experiments (Figure 9A) and cell viability analyses (Figure 9B). NADA did not affect cellular folate concentrations when present in culture medium at 50 μM, but reduced cellular folate concentrations by nearly 75% when present at 75 μM (Table 2). This observation of a threshold NADA concentration suggests that cells can sequester, eliminate or metabolize NADA efficiently but these activities saturate between 50 and 75 μM. Treatment of MCF-7 cells with dopamine or L-dopa had no affect on total intracellular folate concentrations (Table 2), consistent with data from the pulse-chase experiments indicating that NADA accelerates folate turnover (Figure 8). Analysis of MCF-7 cell viability following incubation with various concentrations of NADA indicates that the dose-dependent decreases in cellular [^{3}H]folate content in viable cells parallels NADA-induced cell death (Figure 9B). Neither the addition of dopamine or epinephrine to culture medium impaired [^{3}H]folate accumulation as compared to NADA treatment (Figure 9A). These results indicate that although all catecholamines are capable of undergoing oxidation, only NADA can accelerate folate catabolism and deplete cellular folate in cultured cells.

To investigate potential interactions of NADA and MTHFS in this MCF-7 cell culture model, the effect of NADA treatment on [^{3}H]folate accumulation in cells expressing the MTHFS cDNA (MCF*MTHFS1* cells) was investigated. MCF*MTHFS1* cells display a 2-fold increase in MTHFS activity and are impaired in their ability to accumulate folate; they contain 40% less folate relative to the nontransfected cell line [8]. In contrast to the effects of NADA on folate accumulation in MCF-7 cells, folate accumulation was not impaired in MCF*MTHFS1* cells treated with 0, 25, or 50 μM NADA (Figure 9C). The NADA concentration required to deplete 50% of the radiolabeled folate pool was increased from about 50 μM NADA in MCF-7 cells to about 80 μM NADA in MCF*MTHFS1* cells indicating that MTHFS protects cellular folate pools from NADA-mediated turnover.

DISCUSSION

This study is the first to demonstrate an interaction between folate-mediated one-carbon metabolism and catecholamines. Both recombinant mouse and purified rabbit liver MTHFS were shown to bind oxNADA tightly, non-covalently, and with specificity; neither dopamine nor oxidized dopamine could bind to MTHFS. Catecholamines are highly reactive and known to covalently modify cellular proteins [29], yet oxNADA did not form adducts with the MTHFS protein, as evidenced by its release from MTHFS in the presence of 5-formylTHF. Previous studies of MTHFS protein purified from rabbit and human tissues did not identify a bound catecholamine, but this is likely due to the use of a 5-formylTHF-sepharose affinity column in the protein purification procedure [22,33]. Interestingly, the absorption spectrum for recombinant *Arabidopsis* MTHFS also contains a chromophore that exhibits a shoulder peak in the absorption spectrum at 320 nm similar to that observed for the recombinant mouse MTHFS protein (personal communication, Andrew Hanson), indicating that other recombinant MTHFS proteins also co-purify with a bound catecholamine and that this binding site is conserved among species. Although catecholamines, including NADA, have been demonstrated to be competitive inhibitors of pterin-binding proteins such as sepiapterin reductase [34], the results from this study indicate that MTHFS may have an independent binding site for oxNADA. NADA does not inhibit MTHFS catalytic activity and application of recombinant mouse MTHFS protein to a 5-formylTHF-sepharose column releases tightly-bound oxNADA from the protein. This indicates that NADA and folate do not bind competitively to the same site on MTHFS, but rather suggests that folate binding may induce a conformational change in MTHFS that reduces its affinity for oxNADA.

In cultured cells, NADA increases rates of folate turnover, impairs folate accumulation and depresses intracellular folate concentrations. NADA, when present in culture medium, was the only catecholamine investigated that targeted cellular folate. *In vitro* experiments demonstrated that all reduced folates, with the exception of 5-formylTHF, were rapidly oxidized by oxidized catecholamines (data not shown), indicating that these compounds can catabolize folate. The *ortho*-diphenolic groups present in catecholamines, including NADA, dopamine, L-dopa and epinephrine, undergo oxidation generating *ortho*-quinone species which are short lived and very reactive [28]. Dopamine and NADA *o*-quinones are known to function as two-electron acceptors for a variety of redox reactions *in vitro*, which regenerate the diphenolic ring of the catecholamine [27,30]. There are also numerous examples of proteins that contain quinone cofactors to catalyze oxidative reactions. Copper amine oxidases contain a quino-cofactor, tyrosine-derived 2,4,5-trihydroxyphenylalanyl quinone, and these enzymes catalyze the oxidative deamination of amines to aldehydes [35]. Despite the general reactivity of catecholamines and their ability to oxidize reduced folates, our study indicates that only NADA impairs cellular folate concentrations and increases folate turnover rates.

Previously, we reported that increased MTHFS expression accelerates folate turnover and reduces intracellular folate concentrations [38], although it is not established whether MTHFS

directly catalyzes folate catabolism or if increased rates of folate catabolism result from MTHFS-induced redistribution of the folate one-carbon forms, some of which are labile. Expression of the human MTHFS cDNA in SH-SY5Y cells causes a redistribution of the one-carbon forms of folate and loss of 5-methylTHF, which impairs homocysteine remethylation and imparts a higher methionine requirement for optimal cell growth [20]. In this study, the effect of NADA on cellular folate turnover and accumulation may have resulted from: (1) the binding of oxNADA to MTHFS forming a complex capable of accepting two-electrons for MTHFS-mediated folate catabolism and/or (2) oxNADA acting independently and oxidizing non-protein bound folate. To distinguish between these two possibilities, the ability of the MTHFS-oxNADA complex to oxidize dihydrofolate, 5-methylTHF or 5-formylTHF resulting in the generation of *p*ABG was investigated using both the recombinant murine protein and the purified rabbit liver protein. We were not able to demonstrate *in vitro* folate catabolic activity using either the purified rabbit or mouse MTHFS proteins or reconstituted MTHFS-oxNADA complexes (data not shown). The observation that MCF-7 cells with increased MTHFS expression were less sensitive to NADA treatment provides additional evidence that the MTHFS-oxNADA binary complex does not catabolize folate, but rather protects folate from degradation by oxNADA. In summary, the results from this study demonstrated that MTHFS binds oxNADA or a derivative thereof tightly and this interaction may serve to sequester oxNADA and protect cellular folate from oxNADA-mediated oxidation. Once the cellular MTHFS binding capacity is exceeded, folate may become vulnerable to oxNADA.

NADA is a component of normal human urine (0.5 μmoles/day) and kidney (1.46 nanomoles/gram) [31] but is not found in the human caudate or whole mouse brain, indicating that NADA is formed peripherally [32]. The biological origin and physiological role of NADA is not known, but *N*-acetyltransferase 2 (NAT2) is capable of acetylating dopamine to NADA [31, 32]. Urinary NADA concentrations are elevated in individuals treated with monoamine oxidase inhibitors [36], and children with neuroblastomas and nephroblastomas display 13-fold and 4-fold, respectively, higher levels of urinary NADA compared to unaffected individuals [36, 37]. The effect of elevated NADA on whole-body folate status in these patients has not been examined, although folate deficiency is commonly observed in a number of cancer models [7]. The effects of NADA and potentially other catecholamines on folate status and one-carbon metabolism in an animal model warrants future investigation.

Acknowledgements

The authors would like to thank Jim Kerwin for his help with the collection of mass spectrometry data, and Martha Field for editorial assistance. This work was supported by PHS HD35687 to PJS and DK07158-28 to MCA.

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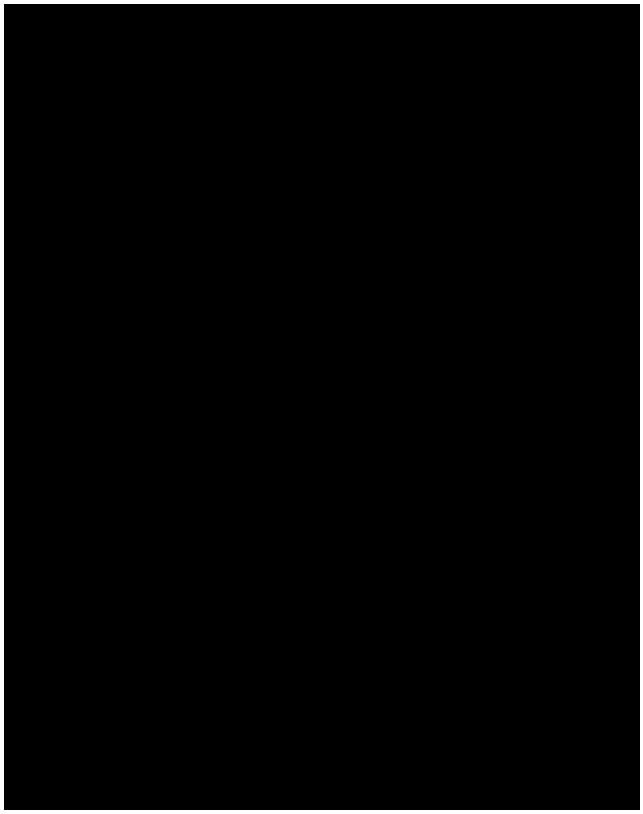

Figure 1. Catabolism of THF

Oxidation of THF results in the loss of two electrons and two protons and formation of a Schiff base between the C^9-N^{10} bond. Hydrolysis of the Schiff base intermediate generates the folate degradation products *para*-aminobenzoylglutamate and tetrahydropterin-6-aldehyde.

Figure 2. UV spectrum of recombinant mouse MTHFS protein

Recombinant mouse MTHFS protein, expressed in *E.coli* BL21 Star™ cells grown in LB medium, was purified using metal affinity chromatography then dialyzed overnight in 100 mM Tris pH 8.0. Recombinant mouse MTHFS protein (solid line) has an absorbance shoulder peak at λ_{max} = 320 nm that is absent in the spectrum of purified rabbit liver MTHFS protein (dashed line).

Figure 3. MALDI-TOF spectrum of recombinant mouse MTHFS protein

The molecular mass of recombinant mouse MTHFS was determined by MALDI-TOF mass spectrometry. A sample of the recombinant mouse MTHFS protein in water was diluted 1:1 with 100% acetonitrile, added to a sinapinic acid matrix, then spotted on the laser target. The recombinant mouse MTHFS protein is present as two predominant species, containing (m/z = 25403) or lacking the chromophore (m/z = 25221). Horse apomyoglobin (m/z = 16951) was added to the MTHFS protein sample as an internal standard. The inset figure is an enlargement of the peaks corresponding to recombinant mouse MTHFS protein.

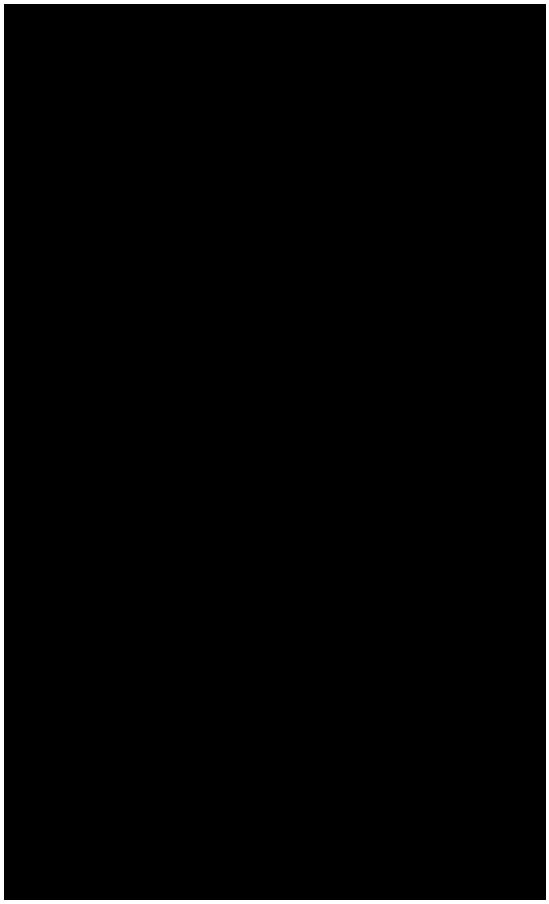

Figure 4. UV spectrum of the chromophore removed from recombinant mouse MTHFS and the spectrum of oxNADA

The chromophore was removed from the recombinant mouse MTHFS protein using a 5-formylTHF-sepharose column equilibrated with 20 mM Tris pH 7.0. Fractions of the flowthrough were collected immediately after the protein was added. *Panel A*: The UV spectrum of the flowthrough fraction (containing the chromophore removed from recombinant mouse MTHFS protein). *Panel B*: The UV spectrum of oxNADA, pH 7.0.

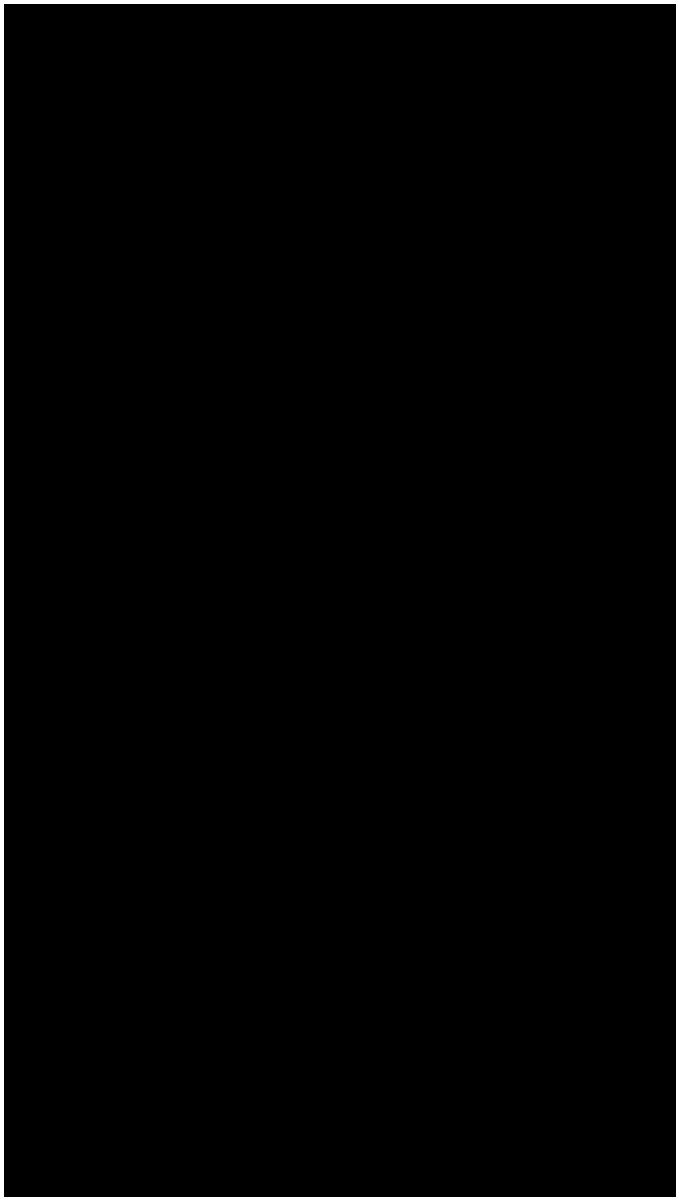

Figure 5. Positive and negative mass spectra for the chromophore removed from the recombinant mouse MTHFS protein

Two different methods were used to remove the chromophore from recombinant mouse MTHFS protein, and each method generated different mass spectra. For spectra A and B, recombinant mouse MTHFS protein was purified using affinity chromatography, then applied to a 5-formylTHF-sepharose affinity column equilibrated with 20 mM 2-mercaptoethanol. Fractions of the flowthrough were collected, and concentrated using a speed vac. *Panel A*: The positive mass spectrum of the chromophore isolated by affinity chromatography. *Panel B*: The negative mass spectrum of the chromophore isolated by affinity chromatography. The peaks labeled with asterisks are an exact match to oxNADA standards. The peaks labeled with crosses differ from oxNADA standards by one atomic mass unit. The spectra for oxNADA standards are not shown. *Panel C*: The positive mass spectrum of the chromophore that was removed from recombinant mouse MTHFS protein by acetonitrile extraction.

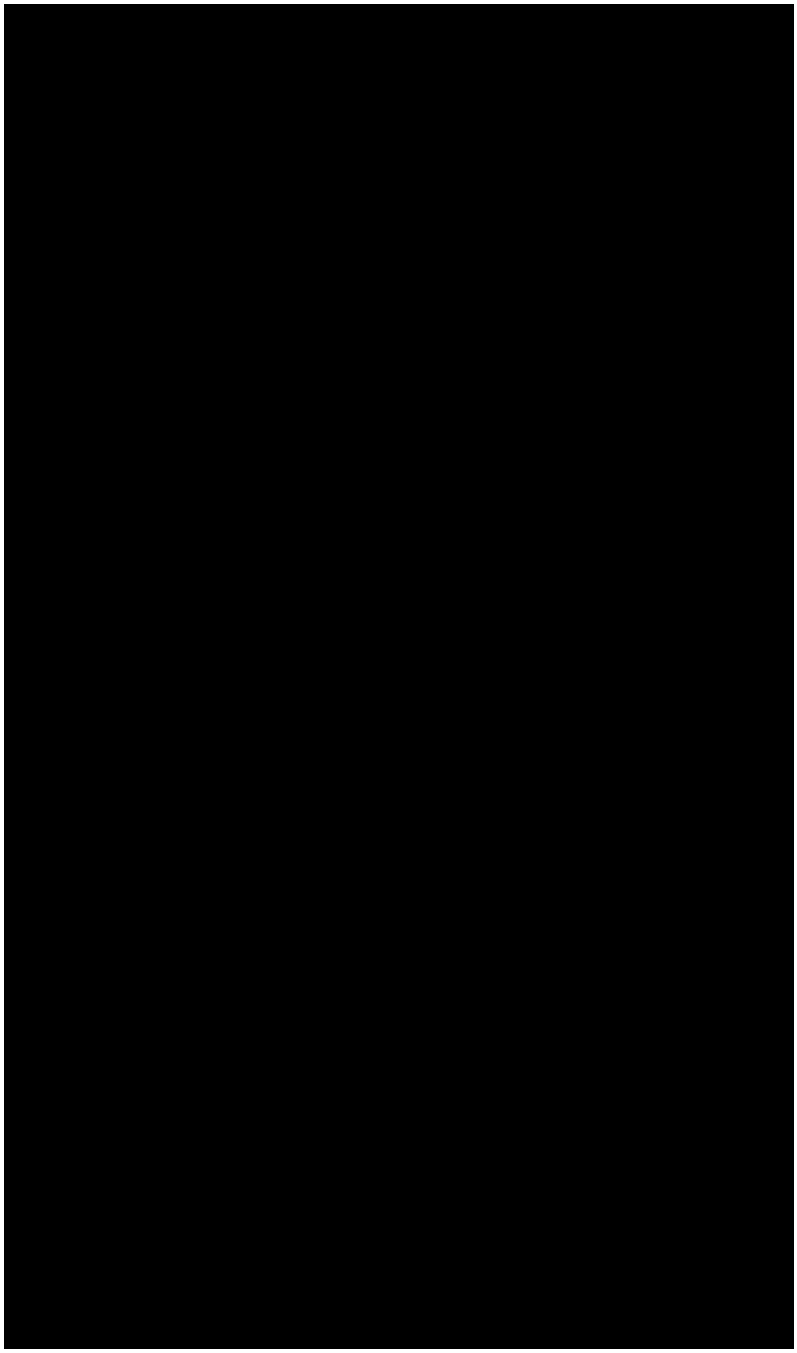

Figure 6. ESI analysis of the chromophore removed from recombinant mouse MTHFS protein expressed in *E.coli* grown in M9 minimal medium containing NADA

E.coli expressing recombinant mouse MTHFS protein were grown in M9 minimal medium, and the MTHFS protein was purified as described previously [26]. The purified mouse MTHFS protein was loaded on a 5-formylTHF-sepharose column and flowthrough fractions were collected and analyzed using ESI-MS. *Panel A*: The spectrum of the flowthrough fractions collected from an MTHFS protein sample that was expressed in *E.coli* grown in M9 medium. *Panel B*: The spectrum of the chromophore (collected as the flowthrough fractions) removed from the MTHFS protein that was expressed in *E.coli* grown in M9 medium supplemented with 10 mg/L NADA.

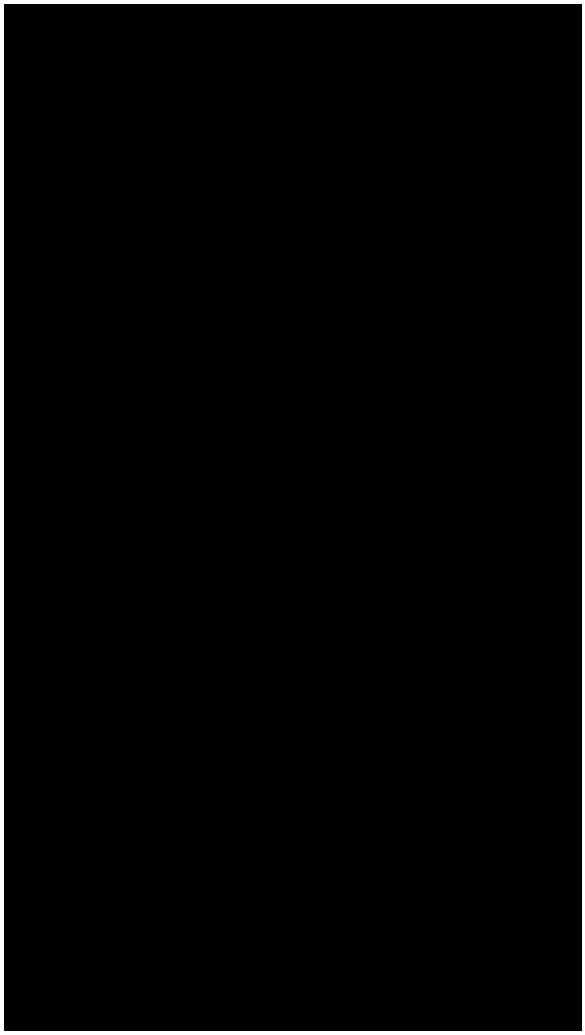

Figure 7. Reconstitution of purified rabbit liver MTHFS with NADA and oxNADA

The UV spectra of purified rabbit liver MTHFS and yeast aldehyde dehydrogenase following incubation with NADA and oxNADA are shown. Stoichiometric amounts of either purified rabbit liver MTHFS or yeast aldehyde dehydrogenase were incubated with NADA (dashed line), oxNADA pH 7.0 (solid line), or a potassium phosphate buffer pH 7.2 (dotted line), then dialyzed overnight. The UV spectra of the proteins were taken after 24 h dialysis. The spectrum of purified rabbit liver MTHFS is shown in *Panel A*, and the spectrum of aldehyde dehydrogenase is shown in *Panel B.*

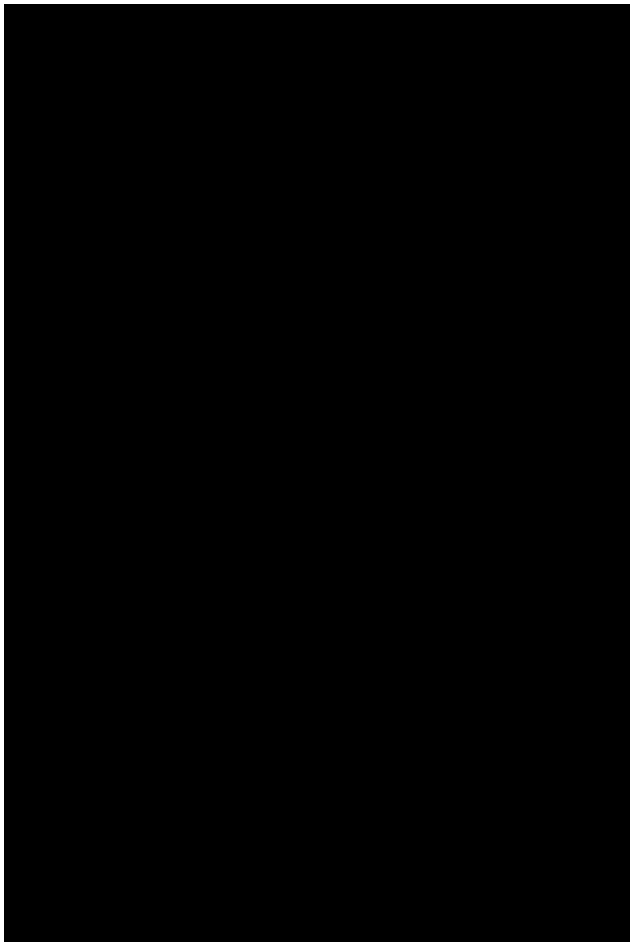

Figure 8. Effect of NADA, dopamine and L-dopa on folate turnover in MCF-7 cells

Cells were labeled with 25 nM [^{3}H]5-formylTHF for 12 h (pulse). The labeled folate was chased with defined αMEM culture medium that contained unlabeled 2 μM folic acid. Total [^{3}H]folate compounds remaining in the cells were determined at various time points. *Panel A:* Squares represent cells chased with 2 μM folic acid, triangles are cells chased with medium containing folic acid and 20 μM dopamine, and inverted triangles are cells chased with medium containing folic acid and 20 μM NADA. *Panel B*: Squares represent cells chased with 2 μM folic acid and triangles represent cells chased with medium containing folic acid and 20 μM L-dopa. All data are expressed as % cpm recovered in cells relative to the total counts recovered in cell lysates and medium at each time point. All values represent triplicate measures and variance is expressed as standard error of the mean.

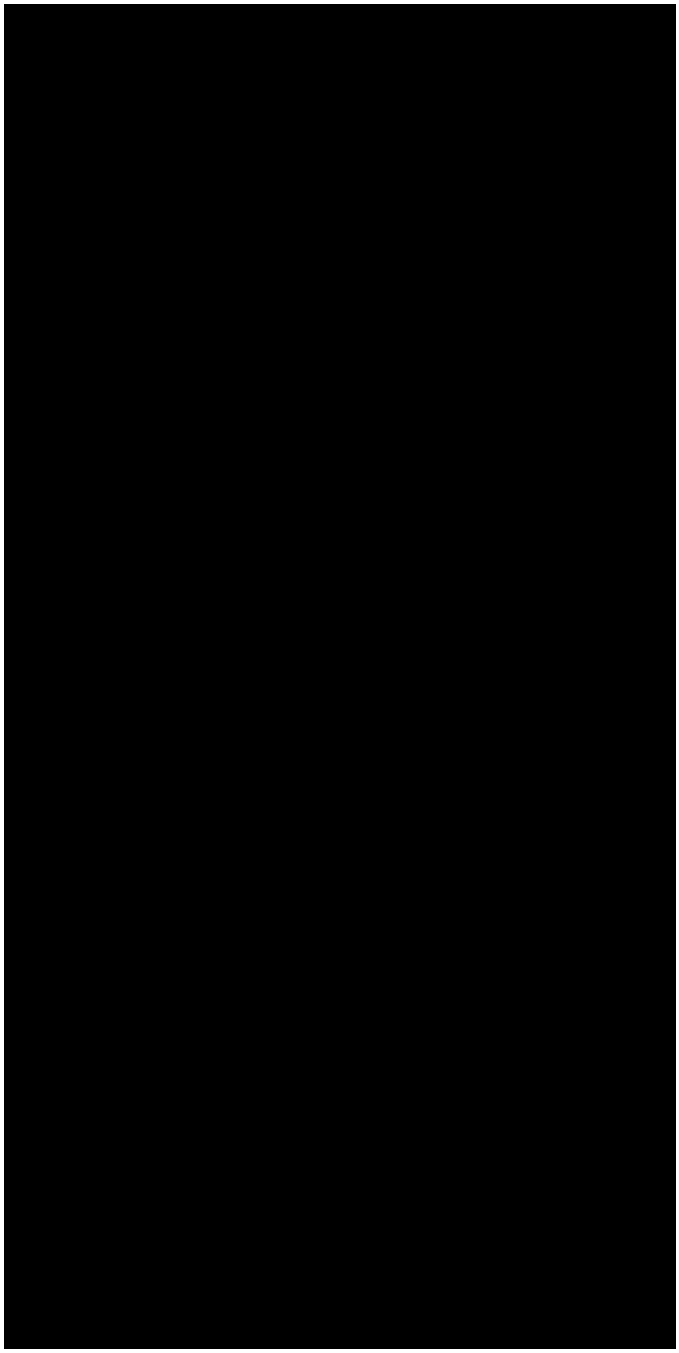

Figure 9. Effect of NADA, dopamine, and epinephrine on folate accumulation and cell viability in MCF-7 cells

MCF-7 cells were incubated with 25.0 nM (6*S*)-[^{3}H]5-formylTHF and various concentrations of NADA, dopamine, or epinephrine for 12 h, then viable cells were quantified using trypan blue staining. *Panel A*: Folate accumulation in MCF-7 cells treated with NADA (inverted triangles), dopamine (squares), and epinephrine (triangles). All values were normalized to cpm for untreated cells, and untreated cells were given an arbitrary value of 100%. *Panel B*: Cell viability (squares) and intracellular [^{3}H]folate accumulation in viable cells (triangles) treated with NADA. Cell viability is expressed as the percent of viable cells divided by the total number of cells. *Panel C:* Comparison of [^{3}H]folate accumulation in MCF-7 (squares) and

MCF*MTHFS* (triangles) cell lines. Total [^{3}H]folate compounds were quantified in cell lysates by liquid scintillation. All values represent quadruplicate measures, and variance is expressed as standard deviations of the mean.

Table 1

Tandem mass spectrometry (ES-MS/MS) analyses of the chromphore removed from recombinant mouse MTHFS and a NADA standard

The predominant peaks (m/z = 197, 213, 363) present in the positive mass spectrum of the chromophore (removed by acetonitrile extraction, Figure 5C) were selected for MS/MS analyses (labeled as "MTHFS"), and the resulting fragmentation peaks are shown. Peaks present in a sample of NADA (m/z = 197, 213, 363) were also fragmented using MS/MS analysis, and the resulting peaks are labeled as "NADA".

MTHFS (m/z = 363)	NADA (m/z = 363)	MTHFS (m/z = 197)	NADA (m/z = 197)	MTHFS (m/z = 213)	NADA (m/z = 213)
363.2	363.2	197	196.3	213.4	213.3
214.4	214.3	179.3	179.3	196.2	197.3
185.4	185.3	167.3	/	185.2	185.3
174.4	174.4	155.2	154.3	/	171.3
146.5	146.4	137.3	137.4	88.6	/
130.5	130.4	123.5	/		

Table 2

Effect of catecholamines on intracellular folate concentrations

MCF-7 cells were cultured in triplicate for 12 h in αMEM medium containing various concentrations of catecholamines. Cells were harvested and intracellular folate concentrations were determined using a microbiological assay. Values represent the means of duplicate measurements, and standard deviation of the mean is shown.

Catecholamine Concentration (μM)	NADA Treatment (fmole folate /μg protein)	Dopamine Treatment (fmole folate /μg protein)	L-dopa Treatment (fmole folate /μg protein)
0	31.8 ± 0.38	32.2 ± 4.02	24.2 ± 0.94
25	32.2 ± 0.97	28.6 ± 3.82	27.7 ± 0.67
50	37.3 ± 7.88	28.6 ± 2.83	30.3 ± 1.26
75	9.9 ± 2.26	28.4 ± 4.33	27.7 ± 0.66
100	7.3 ± 2.77	25.4 ± 2.67	30.0 ± 0.53
150	7.5 ± 2.70	29.5 ± 3.67	27.6 ± 0.92