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Histone Demethylase LSD1 Is a Folate-Binding Protein

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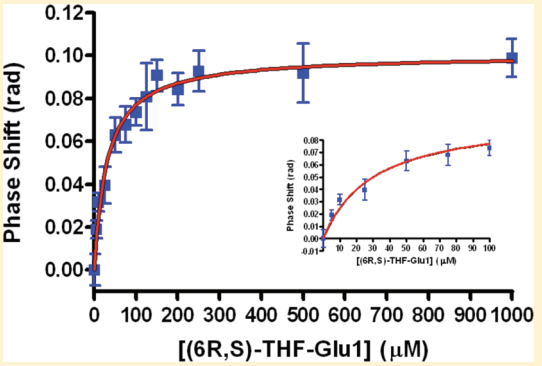
ABSTRACT: Methylation of lysine residues in histones has been known to serve a regulatory role in gene expression. Although enzymatic removal of the methyl groups was discovered as early as 1973, the enzymes responsible for their removal were isolated and their mechanism of action was described only recently. The first enzyme to show such activity was LSD1, a flavin-containing enzyme that removes the methyl groups from lysines 4 and 9 of histone 3 with the generation of formaldehyde from the methyl group. This reaction is similar to the previously described demethylation reactions conducted by the enzymes dimethylglycine dehydrogenase and sarcosine dehydrogenase, in which protein-bound tetrahydrofolate serves as an accepter of the formaldehyde that is generated. We now show that nuclear extracts of HeLa cells contain LSD1 that is associated with folate. Using the method of back-scattering interferometry, we have measured the binding of various forms of folate to both full-length LSD1 and a truncated form of LSD1 in free solution. The 6*R*,*S* form of the natural pentaglutamate form of tetrahydrofolate bound with the highest affinity (K_d = 2.8 μM) to full-length LSD1. The fact that folate participates in the enzymatic demethylation of histones provides an opportunity for this micronutrient to play a role in the epigenetic control of gene expression.



Folate cofactors in eukaryotic cells have been considered to be distributed between both the cytosol and mitochondria where they are used for transfer of one-carbon units between numerous metabolic pathways.[1] All of the natural folate cofactors are polyglutamylated intracellularly and bind to the respective enzymes more tightly than the corresponding forms that contain only a single glutamate residue. In most cases, however, the monoglutamate forms perform the same reaction as the natural, polyglutamated forms.[1] Early studies from our laboratory showed that the polyglutamated form of tetrahydrofolate was tightly bound to the mitochondrial enzymes, dimethylglycine dehydrogenase (DMGDH) and sarcosine dehydrogenase (SDH). Both of these enzymes conduct demethylation reactions in which the *N*-methyl group is first oxidized to the imine followed by hydrolysis to liberate formaldehyde. We showed that bound tetrahydrofolate reacts with the formaldehyde that is generated to produce *N*-5,10-methylenetetrahydrofolate, presumably to protect the enzymes from cross-linking by the formaldehyde formed at the active site.[2−4]

Several early studies showed that, in addition to cytosol and mitochondria, a small amount of folate was also found in the nuclei.[5,6] Earlier studies from our laboratory showed that 24 h after injection of radioactive folate into rats and subsequent analysis of the liver cytosol, mitochondrial, and nuclear fractions, ∼2.5% of the radioactivity was located in the nuclei.[7] The role of folate in the nucleus was unknown until the work of Prem veer Reddy and Pardee.[8] They identified a multienzyme complex that they named "replitase" that contained the enzymes thymidylate synthase and dihydrofolate reductase that used different forms of folate as substrates. They suggested that the replitase complex was involved in the metabolic control of DNA replication. In a recent series of publications, the Stover laboratory reported strong evidence of the nuclear localization of the most important folate-dependent enzymes in the thymidylate biosynthesis pathway: serine hydroxymethyltransferase (SHMT), thymidylate synthase (TS), and dihydrofolate dehydrogenase (DHFR).[9,10] SHMT uses tetrahydrofolate (THF) for synthesis of 5,10-methylene tetrahydrofolate, which is then used by TS for synthesis of thymidylate with dihydrofolate as a product.

The mechanism of histone H3 demethylation by demethylase LSD1 attracted our attention, because of its similarity to the mechanism of demethylation of dimethylglycine by DMGDH and sarcosine by SDH.[2−4,11,12] In all three enzymes, the first step of demethylation is the oxidation of *N*-methyl groups to an imine intermediate. The latter is nonenzymatically hydrolyzed to the demethylated amine and formaldehyde. Most importantly, both DMGDH and SDH contain tightly bound THF, which was shown to form 5,10-methylene tetrahydrofolate, thereby serving as a trap for formaldehyde to prevent cross-linking of proteins.[4]

This, and the preszence of folate in eukaryotic nuclei, suggested that LSD1 might also use tetrahydrofolate to form 5, 10-methylene tetrahydrofolate as a result of histone demethylation. Methylation on the N-terminal tails of histone lysines serves as an epigenetic control mechanism.[13] It should be noted that

Received: February 17, 2011
Revised: April 19, 2011
Published: April 21, 2011

enzymatic demethylation of histones was first reported in 1973,[14] but the enzymes responsible were not isolated. Recently, a group of histone demethylases were discovered in the nucleus.[15−20] There are two classes of these enzymes that perform the removal of a methyl group from different methylated lysine residues in the histones. One class of these lysine-specific demethylases, LSD1 and LSD2, consists of amine oxidases containing FAD as the electron acceptor to first oxidize the *N*-methyl amine to an imine. They catalyze the demethylation of mono- and dimethylated lysine residues, 4 and 9, on histone H3 (H3 K4 and H3 K9, respectively). The second class of histone demethylases is a JmjC family of iron (II)-α-ketoglutarate-dependent histone demethylases for conducting the oxidation of the *N*-methylamine to the imine. The properties of these enzymes are discussed in recent reviews.[19,20]

We hypothesized that LSD1 contains bound polyglutamylated THF that could play a similar role in trapping formaldehyde as in the case of DMGDH and SDH. To test this hypothesis, we studied the localization of folate in nuclei and its association with LSD1 as well as the binding of various forms of tetrahydrofolate with LSD1. We now show that LSD1 is associated with folate in the nuclei of HeLa cells and that various forms of folate bind tightly in vitro to purified LSD1. The natural form of THF polyglutamate bound with the highest affinity.

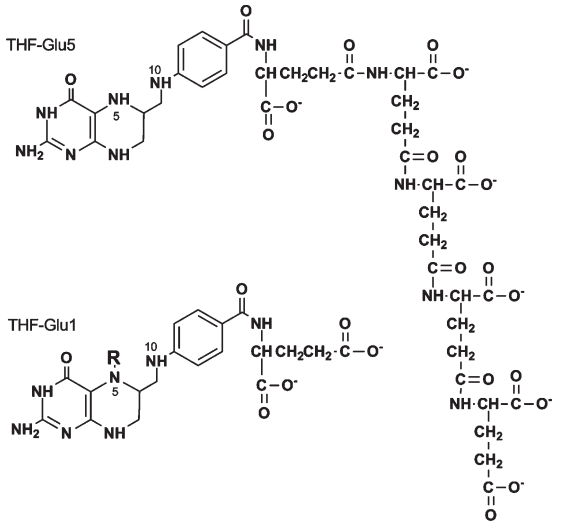


Figure 1. Chemical structures of folates used in the binding study. The monoglutamate and pentaglutamate forms of tetrahydrofolate are shown. In the monoglutamate form, R indicates the position of an H atom at the N-5 position in THF, a CH_3 group in 5-methyl-THF, and a CHO group in 5-formyl-THF.

EXPERIMENTAL PROCEDURES

Materials. Formaldehyde dehydrogenase, peroxidase, NAD, 4-aminoantipurine, 3,5-dichloro-2-hydroxybenzenesulfonic acid, and all general chemicals for buffers and microbiological media were from Sigma. Dimethylhistone H3(Lys4) peptide and antibodies against LSD1 were from Millipore. PreScission protease was from GE Healthcare.

The following were gifts from EPROVA: the stereoisomers of tetrahydrofolate monoglutamate [(6*R*,*S*)-THF-Glu1] and the natural 6*S* stereoisomers of tetrahydrofolate monoglutamate [(6*S*)-THF-Glu1, (6*S*)-5-methyl-THF-Glu1, and (6*S*)-5-formyl-THF-Glu1]. Tetrahydrofolate pentaglutamate [(6*R*,*S*)-THF-Glu5] was synthesized from pteroylpenta-γ-L-glutamic acid (Schircks Laboratory) by reduction with $NaBH_4$ in the presence of $Pb(NO_3)_2$ according to a published procedure.[21,22] The product was spectrophotometrically pure THF (λ_{max} = 298 nm at pH 7.0). It was desalted on a Bio-Gel P-2 column equilibrated with 40 mM ammonium acetate (pH 7.0) and 100 mM β-mercaptoethanol. After the sample had been desalted, β-mercaptoethanol was added to a final concentration of 0.4 M and THF preparations were kept in small aliquots at −20 °C under argon. Solutions of other folates were prepared in 0.4 M β-mercaptoethanol and were kept at −20 °C. Folate concentrations were determined spectrophotometrically in 20 mM potassium phosphate buffer (pH 7.0) and 14 mM β-mercaptoethanol by using the following extinction coefficients: 29.1 mM^{-1} cm^{-1} at 298 nm for all forms of THF, 31.7 mM^{-1} cm^{-1} at 290 nm for (6*S*)-5-methyl-THF-Glu1, and 37.2 mM^{-1} cm^{-1} at 285 nm for (6*S*)-5-formyl-THF-Glu1.[23] The chemical structures of folates used in this work are shown in Figure 1.

The plasmid for full-length LSD1 expression was a generous gift from Y. Shi (Harvard University, Cambridge, MA). In this plasmid, a full-length human cDNA for LSD1 was cloned into the pET-15b expression vector. The cloned cDNA contained an additional sequence at the N-terminus, MGSSHHHHHHSSGLVPRGSNF, which included six histidines and a cleavage site for thrombin (LVPRGS). The predicted molecular mass of that protein with an N-terminal methionine is 95190 Da. By analysis of a tryptic digest of LSD1 using MALDI and LC−MS/MS methods at the Proteomics Facility of the Vanderbilt University Mass Spectrometry Center, it was determined that N-terminal methionine in the expressed protein had been removed; therefore, the molecular mass of the expressed LSD1 was 95059 Da.

The plasmid for the N-terminally truncated LSD1 was a generous gift from P. A. Cole (Johns Hopkins University, Baltimore, MD). In this plasmid, part of LSD1 cDNA (amino acids 171−852) was cloned into a pGEX-6P1 expression vector from which it was expressed as a fusion protein with glutathione *S*-transferase (GST) and a site for PreScission protease.

Methods. *Size-Exclusion Chromatography of Nuclear Protein Extracts.* A nuclear extract (75 μL) from HeLa cells (BIOMOL) containing 695 μg of protein was mixed with column buffer [50 mM CHES, 50 mM HEPES, 100 mM NaCl, and 20 mM β-mercaptoethanol (pH 7.85)] and the mixture applied to a Superose-12 column (Pharmacia) equilibrated with column buffer. Protein was eluted using an AKTA-Purifier system (Amersham) at a flow rate of 0.5 mL/min, and fractions of 0.5 mL were collected. Protein elution was monitored by absorbance at 280 nm. Each fraction was also analyzed for the presence of folate by using a microbiological method (*Lactobacillus casei*[24]) and for the presence of LSD1 by dot blotting with antibodies against LSD1. The Superose column was calibrated with cytochrome *c*, lysozyme, chymotrypsin, carbonic anhydrase, ovalbumin, BSA, alcohol dehydrogenase, potato β-amylase, and aldolase without added urea and also with column buffer containing 8 M urea. The void volume, V_o, was determined with blue dextran, and the total solvent-accessible volume, V_t, was determined with acetone as reported previously.[25]

Full-Length Protein. For expression of full-length LSD1, *Escherichia coli* BL21(DE3) competent cells were transformed with a plasmid containing full-length human LSD1 with an attached N-terminal His tag in the pET-15b expression vector

using a standard heat-shock protocol. It was cultured overnight in 30 mL of LB medium with 100 mg/L ampicillin. The overnight culture was inoculated into 1 L of Terrific Broth containing 100 mg/L ampicillin. It was cultured at 37 °C until the absorbance at 600 nm reached 1.4−1.6. At this time, IPTG was added to a final concentration of 0.5 mM and incubation continued for 7 h at 25 °C. The cells were harvested by centrifugation, washed with cold 0.1 M Tris-HCl (pH 7.5), and kept at −20 °C until the protein was purified.

Purification of Full-Length LSD1. The initial procedure used for purification of full-length LSD1 based on the use of a Ni-agarose affinity chromatography resulted in a relatively low yield.[15] A much better purification of LSD1 could be achieved by combination of ammonium sulfate precipitation and ion-exchange chromatography on DE-52 cellulose. In this protocol, the collected *E. coli* cells were sonicated in homogenization buffer in which the components were 20 mM Tris-HCl buffer (pH 7.8), 14 mM β-mercaptoethanol, and protease inhibitors (protease inhibitor cocktail from Sigma or combination of leupeptin, pepstatin, and PMSF). Cell debris was removed by centrifugation in a Sorvall RC-5 centrifuge in an SS-34 rotor at 18000 rpm for 30 min at 4 °C.

Proteins in the supernatant were fractionated by ammonium sulfate precipitation. The protein fraction that precipitated between 26 and 36% ammonium sulfate saturation contained most of the LSD1 protein. This protein fraction was dissolved in DE-52 buffer [20 mM Tris-HCl (pH 7.8) containing 5 mM β-mercaptoethanol] and desalted on a 20 mL column of Bio-Gel P-30 equilibrated with DE-52 buffer. The desalted protein fraction was loaded onto a 20 mL Whatman DE-52 column equilibrated with DE-52 buffer. The column was washed with DE-52 buffer containing 35 mM NaCl. After being washed, the LSD1 protein was eluted with DE-52 buffer, containing 65 mM NaCl. Fractions containing purified LSD1 were pooled and concentrated using Millipore concentrators (YM-50). This method routinely provided protein with a purity of >96% with a yield of 2.5 mg of LSD1/L of culture. For storage, glycerol was added to LSD1 samples to a final concentration of 40%. This solution was kept at −20 °C in small aliquots and was stable for months without loss of enzyme activity.

N-Terminally Truncated LSD1. Expression of N-terminally truncated LSD1 was similar to that used for the full-length protein with some minor differences in IPTG concentration, temperature, and time of incubation. Collected cells were stored at −20 °C until the protein was purified.

The protocol for protein purification was based on the published method[18] with some substantial differences. Use of ammonium sulfate precipitation greatly reduces the procedure time with no effect on the final enzyme preparation activity. In our protocol, cells were sonicated in homogenization buffer [50 mM HEPES (K salt) (pH 7.8), 150 mM LiCl, 1 μg/mL leupeptin and pepstatin, 1 mM PMSF, 5 mM EDTA, 1 mg/mL lysozyme, 1 μg/mL DNase, and 10 mM DTT]. The homogenate was centrifuged at 18000 rpm in an SS-34 rotor on a Sorvall centrifuge, and the supernatant was fractionated by ammonium sulfate. Proteins that precipitated between 24 and 36% ammonium sulfate saturation were dissolved in the glutathione (GSH) buffer [50 mM HEPES (K salt) (pH 7.8), 150 mM LiCl, 1 μg/mL leupeptin and pepstatin, 0.5 mM PMSF, 5 mM EDTA, and 5 mM β-mercaptoethanol], and the solution was clarified by centrifugation.

The crude extract was loaded on the GSH-agarose (Sigma) column equilibrated with GSH buffer, and unbound proteins were washed out with GSH buffer. The GST-LSD fusion protein was eluted by 30 mM reduced GSH in 50 mM HEPES (Na salt) (pH 8.0). Fractions containing GST-LSD protein were pooled and concentrated to 0.5−1 mL.

GST from the fusion protein was removed by using PreScission protease. The buffer in the sample was first changed to PreScission buffer [50 mM HEPES (Na salt) (pH 7.8), 150 mM NaCl, and 1 mM DTT]. The fusion protein was treated in that buffer with PreScission protease for 16−20 h at 3 °C. After the protease treatment, the N-terminally truncated LSD1 was easily separated from GST and the PreScission protease by a small DE-52 column equilibrated with 25 mM HEPES (Na salt) (pH 7.8). Under these conditions, LSD1 did not bind to the column but other proteins did. The final purity of LSD1 was at least 97% as determined by sodium dodecyl sulfate (SDS) electrophoresis with Coomassie staining.

LSD1 Activity Assay. LSD1 activity was assayed by using coupled assays for hydrogen peroxide or for formaldehyde as described elsewhere.[17] When activity was determined by measuring the level of hydrogen peroxide, the concentrations of the components in the 100 μL reaction mixture were as follows: 25 mM potassium phosphate buffer (pH 7.2), 0.1 mM 4-aminoantipurine, 1.0 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 1 unit of horseradish peroxidase, 17 μM dimethyl-histone H3(Lys4) peptide as a substrate, and 1−3 μM LSD1. The course of reaction was monitored by absorbance at 505 nm in Ultra Micro Cells on a Shimadzu 2401-PC spectrophotometer.

When LSD1 activity was monitored by formaldehyde production, the concentrations of the components in the 100 μL reaction mixture were as follows: 20 mM Tris-HCl (pH 7.5), 17 μM dimethyl-histone H3(Lys4) peptide as the LSD1 substrate, 2 mM NAD, 1−3 μM LSD1, and 0.1 unit of formaldehyde dehydrogenase. The course of reaction was monitored by the increase of absorbance at 340 nm in 70 μL Ultra Micro Cells on a Shimadzu 2401-PC spectrophotometer.

LSD1−Folate Binding. LSD1−folate binding was studied by two methods. Preliminary data were obtained by using separation of unbound folate from a solution of LSD1 and folate with centrifugal devices (concentrators). In initial binding experiments, aliquots of folate solutions were mixed with LSD1 in a total volume of 300 μL and incubated at room temperature in the dark for 1 h. Controls were prepared with the same volume of folate solutions in the protein buffer but without LSD1. After incubation, the LSD1−folate reaction mixtures were loaded on centrifugal filters (Centricon YM-50, Millipore) and centrifuged in an Eppendorf microcentrifuge at 5000 rpm for 10−13 min, yielding 100−120 mL of filtrate. Control samples were treated the same way. Concentrations of various folates in the filtrates were determined by absorption spectra in the 240−400 nm range on a Shimadzu 2401-PC spectrophotometer using Ultra Micro Cells (Shimadzu) with a 70 μL working volume.

Measurement of LSD1−Folate Binding by Back-Scattering Interferometry (BSI). The majority of the binding experiments were performed using BSI, described previously.[26] Briefly, the instrument is composed of a simple optical train that consists of a HeNe laser, a microfluidic chip with a channel etched in borosilicate glass, and a CCD camera. The laser impinges on the samples in the microfluidic channel, producing a high-contrast interference pattern, which is reflected onto the CCD array. Changes in the refractive index (RI) of the solution contained within the channel cause spatial shifts in the interference pattern, which are monitored and recorded using software designed in-house.

Solutions of LSD1 and folates used in BSI experiments were prepared in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 35 mM β-mercaptoethanol, concentrations required to prevent oxidation of oxygen-labile forms of folate. All solutions were filtered through a 0.2 μm filter and degassed prior to binding experiments.

Binding experiments were performed in an end point format.[27,28] For the binding samples, a constant amount of LSD1 was mixed off-line with increasing concentrations of folates and incubated at room temperature in the dark for at least 2 h. This assures that equilibrium has been reached prior to the samples being injected into the BSI instrument.

Specific binding is quantified in BSI experiments by measuring the difference in the signal between the control or blank and the binding pair. Here each form of folate served as its own blank; therefore, a calibration curve of concentration versus BSI signal for the ligand alone was constructed, by preparing samples over the same concentration range that was used in the binding experiments. Then these values were used to correct the binding curves for bulk RI changes that occur due to the increasing concentration of the folates, particularly at higher ligand concentrations.

The difference between the calibration and binding signals was plotted versus ligand concentration, yielding a saturation binding isotherm. The corrected binding data were analyzed with GraphPad Prism (GraphPad Software Inc., San Diego, CA). A one-site binding algorithm was chosen because LSD1 is a monomer that contains a single bound FAD. Therefore, it seems logical that there is one THF binding site per LSD1 molecule. To test this, we performed a statistical comparison of the one-site binding and two-site binding models for the natural ligand, THF-Glu5, using both an F-test and an Akaike's information criterion (AIC) test. Both tests supported the one-site binding model (94.0% confidence using the F-test and 99.3% confidence using the AIC test). This was further supported by the approximately 10-fold increase in the standard error of the calculated K_d values when the two-site binding model was applied. Using the one-site binding model, the K_d was found to be 2.77 $\pm$ 0.46 μM, while the two-site model calculates K_{d1} to be 0.00 $\pm$ 4.39 μM and K_{d2} to be 3.77 $\pm$ 4.96 μM. The same trend was observed for the other forms of folate.

Folate Assay. Folate was assayed by using the *L. casei* method as described by Horne et al.[24] Briefly, the protein samples were mixed with 10 volumes of extraction buffer [50 mM HEPES, 50 mM CHES (pH 7.85), 28 mM β-mercaptoethanol, and 2% ascorbate] and heated for 10 min in boiling water. Precipitated proteins were separated by centrifugation, and the supernatant was treated with conjugase for 3 h at 37 °C. Reaction mixtures were boiled again and centrifuged, and the supernatant was used for analysis according to the original protocol.

Other Methods. The concentration of protein samples was determined by the BCA method (BCA Protein Assay kit, Pierce) with bovine serum albumin as a standard. Protein spectra were recorded on a Shimadzu 2401-PC spectrophotometer. Protein purity was determined by SDS electrophoresis with Coomassie staining. Immunoblotting was conducted according to a standard immunoblotting protocol using a nitrocellulose membrane, primary and secondary antibody binding, and visualization using a SuperSignal West Femto Maximum Sensitivity Substrate Kit (Pierce).

RESULTS

Presence of Folate and LSD1 in Nuclear Protein Extracts from HeLa Cells. HeLa cells have been used as a source for purification of histone demethylases.[29] Therefore, we used nuclei from HeLa cells to look for the possible presence of complexes of LSD1 and folate coenzymes. A commercial preparation of dialyzed nuclear extract (BIOMOL) was fractionated by size-exclusion chromatography. Measurement of total folate in the extract indicated that it contained 2.2 nmol of total folate/mg of nuclear protein. The extract was applied to a Superose-12 column as described in Methods. Elution was monitored by absorbance at 280 nm; fractions were assayed for the presence of folate by a microbiological assay and for the presence of LSD1 by an immunoassay. The results are shown in Figure 2. Eluted proteins were detected in almost all fractions, from the void volume (V_o, 8.0 mL) to the total liquid volume (V_t, 20.1 mL). Most of the folate eluted in a broad band from 16 to 22 mL, indicating it was unbound folates. Because the nuclear extract had been dialyzed prior to chromatography, this indicated that most of the folates that eluted between 16 and 22 mL either had been loosely protein-bound and then dissociated during passage through the column or had been unbound in the nuclear extract and not removed by dialysis. Control experiments showed that THF pentaglutamate (961 Da) eluted from this column at 17.5 mL. This corresponds to a globular protein of ~10 kDa [cytochrome *c* (12400 Da) eluted at 16 mL]. Because of the asymmetry of the folates, they behave in solution as larger molecules and require dialysis membranes with a larger pore size through which to pass. It is therefore highly likely that all folate polyglutamates were not removed by dialysis prior to passing through the Superose column because the preparation from BIOMOL was dialyzed using a membrane with a 10 kDa cutoff. In addition to the major folate peak, there was a smaller folate peak at 12−15 mL. This corresponds to the elution volume of globular proteins of 30−100 kDa as determined by calibration of the column. The folate eluted in this region was probably tightly protein bound.

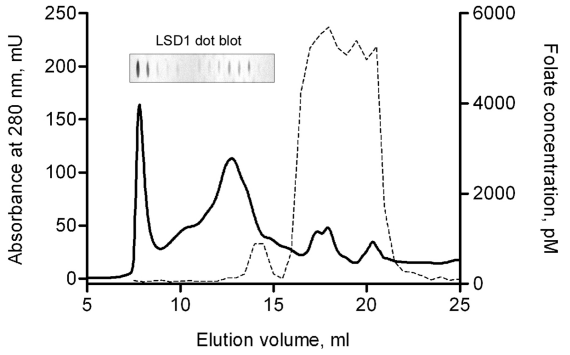


Figure 2. Folate and LSD1 in HeLa nuclear extract. The solid line represents the absorbance at 280 nm. Fractions of 0.5 mL were collected. The dashed line represents the concentration of folate in each fraction. The presence of LSD1 in the fractions was determined immunologically by dot blot. This is positioned to coincide with the fractions that were collected and are an indication of the presence of LSD1 in the eluted fractions.

The presence of LSD1 in these fractions was determined immunologically and appears most abundantly in the void volume where multimeric protein complexes of LSD1 would be found.[16] A smaller but clearly distinct amount of LSD1 was eluted at 12−13 mL that contained the shoulder of the first peak of the early eluting folate. Globular proteins of 100−120 kDa would elute in this region, and LSD1 appears to be ~100 kDa as

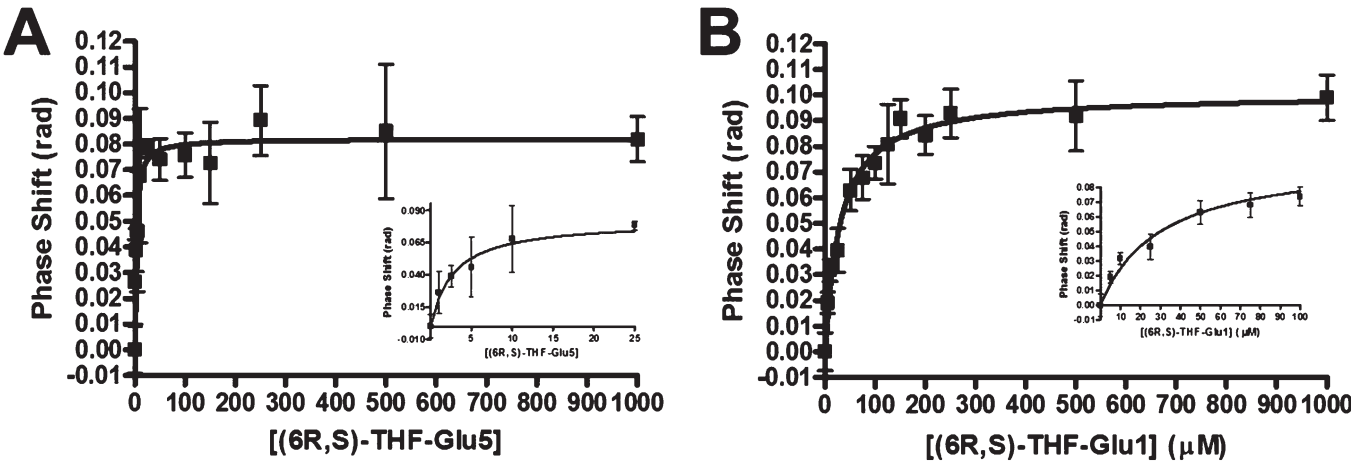


Figure 3. Representative binding curves obtained by BSI. The BSI signal is plotted vs ligand concentration for (A) (6*R*,*S*)-THF-Glu1 and (B) (6*R*,*S*)-THF-Glu5.

determined by SDS electrophoresis. These data are interpreted to indicate that LSD1 in the nuclear protein extract exists mainly as a part of a multimeric protein complex but also as a free protein. An important conclusion is that fractions in which free LSD1 is eluted also contain folate (Figure 2). It is reasonable to speculate that an LSD1−folate complex eluted at this volume. To verify this conclusion, we conducted a series of experiments to measure the in vitro binding of selected forms of folate to LSD1.

LSD1−THF Interaction under Steady-State Conditions. Interaction of folates with LSD was studied by two methods using, first, membrane separation of free folate and, second, BSI as described in Methods. Preliminary data, using membrane separation, indicated an LSD1−folate interaction with very high binding constants (data not shown). After LSD1−folate binding had been established using this method, we used the more sophisticated BSI method for a detailed characterization of LSD1−folate interaction in solution without any physical separation methods, eliminating the potential of a biased result due to nonequilibrium conditions.

In both methods, binding was analyzed in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 35 mM β-mercaptoethanol. That concentration of reducing agent was established in separate experiments to be sufficient to protect THF from oxidation for at least for 6 h as determined spectrophotometrically.

We used four forms of folate [THF-Glu1, THF-Glu5, 5-formyl-THF-Glu, and 5-methyl-THF-Glu1 (Figure 1)] and determined the relative binding affinities for these species. Typical binding isotherms are shown in Figure 3. The first form used was a mixture of stereoisomers, (6*R*,*S*)-THF-Glu5. It was found that the K_d for that form of folate was 2.8 μM (Table 1). To determine whether polyglutamation of THF affects affinity, we studied binding of (6*R*,*S*)-THF-Glu1 to LSD1. Indeed, the binding affinity of the monoglutamate form was significantly lower than that of the polyglutamate species with a K_d value of 30 μM, as compared to a K_d of 2.8 μM.

If binding of THF to LSD1 is specific (indicating biological function), then two things should be observed. First, the natural isomer, (6*S*)-THF, should bind to LSD1 with a higher affinity. We verified that by analysis of the interaction of LSD1 with (6*S*)-THF-Glu1. It was found (Table1) that the affinity of the natural 6*S* isomer is greater, with a K_d almost 2-fold lower (19 μM) than that of the 6*R*,*S* mixture (30 μM).

Table 1. Binding of Different Forms of Folate to LSD1[a]

folate	K_d (μM)
(6*R*,*S*)-THF-Glu5	2.77 ± 0.46
(6*R*,*S*)-THF-Glu1	30.3 ± 3.8
(6*S*)-THF-Glu1	19.5 ± 5.0
(6*S*)-5-CH_3-THF-Glu1	46.3 ± 12.3
(6*S*)-5-CHO-THF-Glu1	72.9 ± 16.2
(6*R*,*S*)-THF-Glu5 with truncated LSD1	4.02 ± 0.92
(6*S*)-5-CHO-THF-Glu1 without BME	65.1 ± 14.0

[a] Values are the means ± the standard error of the mean of dissociation constants.

There are a variety of folate coenzymes that participate in the transfer of one-carbon units, but only THF has the ability to combine nonenzymatically with formaldehyde. A second indication of biological significance would be that these other forms of folate would bind to LSD1 with a lower affinity. To confirm this, we performed folate−LSD1 binding experiments with (6*S*)-5-formyl-THF-Glu1. As expected, the affinity of LSD1 for (6*S*)-5-formyl THF-Glu1 was much lower than that for (6*S*)-THF-Glu1 with a K_d value of 70 μM (Table 1). The order of affinity for the folate species with full-length LSD is as follows: (6*R*,*S*)-THF-Glu5 > (6*S*)-THF-Glu1 > (6*R*,*S*)-THF-Glu1 > (6*S*)-5-CH_3-THF-Glu1 > (6*S*)-5-formyl-THF-Glu1.

An important methodological question in the study of LSD1−THF interaction is whether the use of a high concentration of reducing reagent to prevent the extremely labile THF from being oxidized could influence the interaction. The high concentration (35 mM) of β-mercaptoethanol used in our study also might affect the conformation of LSD1 that could ultimately affect the interaction with folate. We addressed this question by comparing binding of (6*S*)-5-formyl THF-Glu1, which is completely stable in air, to LSD1 in the presence and absence of β-mercaptoethanol. It was found that use of 35 mM β-mercaptoethanol does not change the K_d value for (6*S*)-5-formyl-THF-Glu1 binding (73 μM in the presence and 65 μM in the absence of β-mercaptoethanol).

Having established that (6*R*,*S*)-THF-Glu5 binds with high specificity, we found it was important to identify the binding site in the protein. A definitive answer to this question can be

obtained with a high-resolution crystal structure, work that is in progress in our laboratory. While it is less definitive, some insight into the binding site of folate can be gained by analysis of the interaction of folate with selectively mutated LSD1 species that have been used to obtain a crystal structure of the enzyme. Here, we compared (6*R*,*S*)-THF-Glu5 binding to full-length LSD1 with binding to its N-terminally truncated variant lacking the 170 N-terminal amino acid residues. Results from this binding study showed that the N-terminally truncated protein binds (6*R*,*S*)-THF-Glu5 with the same affinity as the full-length protein (Table 1), indicating that the first 170 amino acid residues do not participate in folate binding.

DISCUSSION

The data presented here indicate that LSD1 is present in nuclei obtained from HeLa cells. It appears to be present primarily as a complex.[16] There is also a small amount of LSD1 that is not present in a complex but appears to be associated with a folate coenzyme. Binding experiments using recombinant full-length LSD1 showed that THF pentaglutamate binds with high affinity, suggesting that the form of folate associated with LSD1 in HeLa cell nuclei was THF pentaglutamate. Furthermore, it is shown that BSI can be used to rank the folate species according to their binding affinity. This is the first time that direct binding, label-free and in solution, has been quantified for this class of molecules. We believe that the biological function of the bound folate is to serve as an acceptor of the formaldehyde that is generated during the oxidative demethylation of histones. Formaldehyde reacts with THF nonenzymatically producing 5,10-CH_2-THF;[30,31] therefore, there is a little doubt that the formaldehyde released in the course of the histone demethylation reaction by LSD1 will react with THF. This would be similar to the role played by THF in similar reactions conducted by dimethylglycine dehydrogenase and sarcosine dehydrogenase.[2−4] The function of THF is to serve as a carrier of one-carbon units that are transferred between enzymes for use as building blocks in a number of metabolic pathways.[1,32] In the nuclei of mammalian cells, at least one metabolic pathway that uses THF has recently been established. The Stover lab has provided strong evidence that in the nuclei a pathway for thymidylate synthesis utilizes THF. In the first step of this synthetic pathway, it is thought that serine hydroxymethyltransferase transfers a methylene group from serine to tetrahydrofolate producing methylenetetrahydrofolate (5,10-CH_2-THF). Once formed, 5,10-CH_2-THF is used by thymidylate synthase for synthesis of dTMP. It would be reasonable to suggest that 5,10-CH_2-THF synthesized by oxidative demethylation might be used as a substrate for thymidylate synthase as well.

The fact that folate participates in the enzymatic demethylation of histones provides an opportunity for this micronutrient to play a role in the epigenetic control of gene expression.

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Funding Sources

Support for this study was, in part, provided by National Institutes of Health Grants DK15289 and DK080010 to C.W. and National Science Foundation Grant CHE-0848788 to D.J.B. and F.M.

ACKNOWLEDGMENT

We thank Dr. Yang Shi and Dr. Philip A. Cole for the generous gift of plasmids for LSD1. We thank EPROVA for the generous gift of folates.

ABBREVIATIONS

LSD1, lysine-specific histone demethylase 1; BSI, back-scattering interferometry; SHMT, serine hydroxymethyltransferase; TS, thymidylate synthase; DHFR, dihydrofolate reductase (folate refers to the general class of folate cofactors); THF, tetrahydrofolate; THF-Glu1, tetrahydrofolate monoglutamate; 5-methyl-THF-Glu1, 5-methyltetrahydrofolate monoglutamate; 5-formyl-THF-Glu1, 5-formyltetrahydrofolate monoglutamate; THF-Glu5, tetrahydrofolate pentaglutamate; JmjC, Jumonji-containing; DMGDH, dimethylglycine dehydrogenase; SDH, sarcosine dehydrogenase.

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