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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 carried out 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 (BSI) we have measured the binding of various forms of folate to both full length LSD1 and to a truncated form of LSD1 in free solution. The (R,S) form of the natural pentaglutamate form of tetrahydrofolate bound with the highest affinity (Kd = 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.

Keywords

lysine-specific demethylase; formaldehyde; tetrahydrofolate; tetrahydrofolate pentaglutamate; ligand binding; back-scattering interferometry

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 carry out demethylation reactions in

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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) (3) (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 hr. after injection of radioactive folate into rats and subsequent analysis of the liver cytosol, mitochondrial and nuclear fractions, about 2.5% of 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” which 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 for 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, due to its similarity to the mechanism of demethylation of dimethylglycine by DMGDH and sarcosine by SDH (2) (3) (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 non-enzymatically hydrolyzed to the de-methylated 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, plus the presence of folate in eucaryotic 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 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) (16) (17) (18) (19) (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, are amine oxidases containing FAD as 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). The second class of histone demethylases is a JmjC family of iron (II)-α-ketoglutarate-dependent histone demethylases to carry out the oxidation of the N-methyl amine to the imine. The properties of these enzymes are discussed in recent reviews (19) (20).

We hypothesized that LSD1 contains bound polyglutamylated THF which could play a similar role to trap 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.

Experimental Procedures

Materials

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

The following were a gift from EPROVA (Switzerland): the stereoisomers of tetrahydrofolate monoglutamate (6R,S)-THF-Glu1, the natural (6S)-stereoisomers of tetrahydrofolate monoglutamate (6S)-THF-Glu1), (6S)-5-Methyl-THF-Glu1 and (6S)-5-Formyl-THF-Glu1. Tetrahydrofolate pentaglutamate (6R,S)-THF-Glu5) was synthesized from pteroylpenta-γ-L-glutamic acid (Schircks Laboratory) by reduction with $NaBH_4$ in 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 desalting, β-mercaptoethanol was added to a 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 K-phosphate buffer, pH 7.0–14 mM β-mercaptoethanol by using extinction coefficients (in $mM^{-1}cm^{-1}$ units): 29.1 at 298 nm for all forms of THF, 31.7 at 290 nm for (6S)-5-Methyl-THF-Glu1 and 37.2 at 285 nm for (6S)-5-Formyl-THF-Glu1 (23). The chemical structures of folates used in this work are shown in Fig. 1.

The plasmid for full-size LSD1 expression was a generous gift of Dr. Shi (Harvard University). In this plasmid a full-size human cDNA for LSD1 was cloned into pET-15b expression vector. The cloned cDNA contained an additional sequence at the N-terminal, MGSSHHHHHHSSGLVPRGSNF, which included 6 histidines and a cleavage site for thrombin (LVPRGS). The predicted molecular mass of that protein with N-terminal methionine is 95,190 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 and therefore the molecular mass of expressed LSD1 was 95,059 Da.

The plasmid for the N-terminal truncated LSD1 was a generous gift of Dr. Cole (Johns Hopkins University). In this plasmid, part of LSD1 cDNA (171–852 aa) 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 extract—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, 20 mM β-mercaptoethanol, pH 7.85) and applied to a Superose-12 column (Pharmacia) equilibrated with Column Buffer. Protein was eluted using AKTA-Purifier system (Amersham) with 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 beta-amylase and aldolase without added urea and also in 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 earlier (25).

1. Full-size protein: For expression of full-size LSD1, *Escherichia coli* BL21 (DE3) competent cells were transformed with a plasmid containing full-size human LSD1 with an attached N-terminal His-tag in pET-15b expression vector using a standard heat-shock protocol. It was cultured overnight in 30 ml of LB media 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 a value of 1.4–1.6. At this time IPTG was added to a concentration of 0.5 mM and incubation continued for 7 hrs 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 protein purification.

Full-size LSD1 purification: The initial procedure used for purification of full-size 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). Cells debris was removed by centrifugation in a Sorvall RC-5 centrifuge in a SS-34 rotor at 18,000 rpm for 30 min at 4°C.

Proteins in the supernatant were fractionated by ammonium sulfate precipitation. The protein fraction precipitated between 26–36% of 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 of 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 washing, the LSD1 protein was eluted by 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 greater than 96% with a yield of 2.5 mg LSD1 from 1 liter of culture. For storage, glycerol was added to LSD1 samples to a final concentration of 40%. This solution was kept at −20° in small aliquots and was stable for months without loss of enzyme activity.

2. N-terminal truncated LSD1: Expression of N-terminal truncated LSD1 was similar to that used for the full-size protein with some minor differences in IPTG concentration, temperature and time of incubation. Collected cells were stored at −20° C until protein purification.

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 final enzyme preparation activity. In our protocol, cells were sonicated in the Homogenization Buffer: 50 mM HEPES (K-salt), pH 7.8, 150 mM LiCl, 1 μg/ml of leupeptin and pepstatin, 1 mM PMSF, 5 mM EDTA, 1 mg/ml lysozyme, 1μg/ml DNase, 10 mM DTT. The homogenate was centrifuged at 17,000 rpm in a SS-34 rotor on a Sorvall centrifuge and the supernatant was fractionated by ammonium sulfate. Proteins precipitated between 24–36% of ammonium sulfate saturation were dissolved in the glutathione (GSH)-Buffer: 50 mM HEPES (K-salt), pH 7.8, 150 mM LiCl, 1μg/ml of leupeptin and pepstatin, 0.5 mM PMSF, 5 mM EDTA, 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 un-bound proteins were washed out by 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, 1 mM DTT. The fusion protein was treated in that buffer with PreScission Protease for 16–20 hrs at 3°. After protease treatment the N-terminal truncated LSD1 was easily separated from GST and the PreScission protease by a small DE-52 column equilibrated with 25 mM HEPES (Na), 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 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 hydrogen peroxide the concentration of the components in reaction mixture of 100 µl was: 25 mM K-phosphate buffer pH 7.2, 0.1 mM 4-aminoantipurine, 1.0 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 1 U of horseradish peroxidase, 17 µM of dimethyl-histone H3(Lys4) peptide as substrate and 1–3 mM 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 concentration of the components in a 100 µl reaction mixture was: 20 mM Tris-HCl pH 7.5, 17 µM of dimethyl-histone H3(Lys4) peptide as LSD1 substrate, 2 mM NAD, 1–3 mM of LSD1 and 0.1 U of formaldehyde dehydrogenase. The course of reaction was monitored by increase of absorbance at 340 nm in 70 µl Ultra Micro Cells on 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-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 hour. 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 to obtain 100–120 ml of filtrate. Control samples were treated the same way. Concentration of various folates in the filtrates was 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, microfluidic chip with 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 refractive index (RI) of the solution contained within the channel cause spatial shifts in the interference pattern, which are monitored and recorded using in-house designed software.

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 concentrations of β-mercaptoethanol required to prevent

oxidation of oxygen-labile forms of folate. All solutions were filtered through 0.2 μm filter and degassed prior to binding experiments.

Binding experiments were performed in an endpoint 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 hours. 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 signal between the control or blank and the binding pair. Here each form of folate served as its own blank, therefore a calibration curve in concentration vs. BSI signal for the ligand alone was constructed, by preparing samples over the same concentration range as 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 concentrations of the ligand.

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 software (GraphPad Software Inc., San Diego, California, USA). 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. In order to test this, statistical comparison of the one-site binding and two-site binding models for the natural ligand, THF-Glu5, was performed 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 ten-fold increase in the standard error of the calculated KD values when the two-site binding model was applied. Using the one-site binding model, the Kd was found to be 2.77 ± 0.46 μM, while the two-site model calculates Kd1 to be 0.00 ± 4.39 μM and Kd2 to be 3.77 ± 4.96 μM. The same trend was observed for the other forms of folate.

Folate assay—Folate was assayed by using the *Lactobacillus 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, 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 hours at 37°. Reaction mixtures were boiled again, 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 Shimadzu 2401-PC Spectrophotometer. Protein purity was determined by SDS electrophoresis with Coomassie staining. Immunoblotting was done according to a standard immunoblotting protocol with using a nitrocellulose membrane, primary and secondary antibodies binding and visualization by using SuperSignal West Femto Maximum Sensitivity Substrate Kit (Pierce).

Results

Presence of folate and LSD1 in nuclear protein extract 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 between 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 nmoles of total folate/mg nuclear protein. The extract was applied to a Sepharose-12 column as described under Methods. Elution was monitored by absorbance at 280 nm; fractions were assayed for the presence of folate by microbiological assay and for the presence of LSD1 by immunoassay. The results are shown in Fig. 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 they were unbound folates. Because the nuclear extract had been dialyzed prior to chromatography, this indicated that most of the folates eluted between 16–22 ml had either been loosely protein-bound and then dissociated during passage through the column or had been unbound in the nuclear extract and not been 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 about 10 kDa (cytochrome C (12,400 Da) eluted at 16 ml). Because of the asymmetry of the folates they behave in solution as larger molecules and require dialysis membranes with larger pore size to pass through. It is therefore highly likely that all folate polyglutamates were not removed by dialysis prior to passage through the Superose column since 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.

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 about 100 kDa by SDS electrophoresis. These data are interpreted to indicate that LSD1 in the nuclei 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 (Fig. 2). It is reasonable to speculate that an LSD1-folate complex eluted at this volume. In order to verify this conclusion we carried out a series of experiments to measure the *in vitro* binding of selected forms of folate to LSD1.

LSD1-THF interaction at 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 was 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 non-equilibrium conditions.

In both methods, binding was analyzed in 50 mM Tris-HCl, pH 7.5–100 mM NaCl-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 hours as determined spectrophotometrically.

We used four forms of folate; THF-Glu1, THF-Glu5, 5-formyl-THF-Glu and 5-methyl-THF-Glu1 (Fig. 1) and determined the relative binding affinities for these species. Typical binding isotherms are shown in Fig. 2. The first form used was a mixture of stereoisomers, (6R,S)-THF-Glu5. It was found that the K_d for that form of folate was 2.8 μM (Table 1). To find out whether polyglutamation of THF affects affinity we studied binding of (6R,S)-THF-

Glu1 with LSD1. Indeed, the binding affinity of monoglutamate form was significantly lower than that of the polyglutamate species with a K_d value of 30 μM, as compared to 2.8 μM.

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

There are a variety of folate coenzymes that participate in the transfer of one-carbon units, but only THF has the ability to combine non-enzymatically with formaldehyde. A second indication of biological significance would be that these other forms of folate would bind to LSD1 with lower affinity. To confirm this we performed folate/ LSD1 binding experiments with (6S)-5-formyl THF-Glu1. As expected, the affinity of LSD1 for (6S)-5-formyl THF-Glu1 was much lower compared to (6S)-THF-Glu1 with a K_d value of 70 μM (Table 1). The order of affinity for the folate species with the full-length LSD is as follows: (6R,S)-THF-Glu5 > (6S)-THF-Glu1 > (6R,S)-THF-Glu1 > (6S)-5-CH_3-THF-Glu1 > (6S)-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 oxidation could influence the interaction. The high concentration (35 mM) of β-mercaptoethanol used in our study also might affect the conformation of LSD1 which could ultimately affect interaction with folate. We addressed this question by comparing binding of (6S)-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 concentration of β-mercaptoethanol does not change the K_d value for (6S)-5-formyl THF-Glu1 binding (73 μM in the presence and 65 μM in the absence of β-mercaptoethanol).

Having established that (6R,S)-THF-Glu5 binds with high specificity, it is important to determine 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 less definitive, some insight into the binding site of folate can be gained by analysis of folate interaction with selectively mutated LSD1 species that have been used to obtain a crystal structure of the enzyme. Here, we compared (6R,S)-THF-Glu5 binding to full-size LSD1 with binding to its N-terminal truncated variant lacking the N-terminal 170 amino acids residues. Results from this binding study showed that the N-terminal truncated protein binds (6R,S)-THF-Glu5 with the same affinity as the full-size 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 non-enzymatically producing 5,10-CH_2-THF (30) (31) therefore there is a little doubt that the formaldehyde released in the course of histone demethylation reaction by LSD1 will react with THF. This would be similar to the role played by THF in similar reactions carried out by dimethylglycine dehydrogenase and sarcosine dehydrogenase (2–4). The function of THF is to serve as a carrier of one-carbon units which 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 been recently 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.

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-formyl-tetrahydrofolate monoglutamate
THF-Glu5	tetrahydrofolate pentaglutamate
JmjC	Jumonji-containing
DMGDH	dimethylglycine dehydrogenase
SDH	sarcosine dehydrogenase

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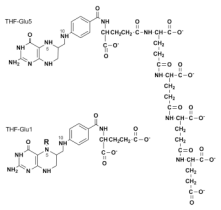

Figure 1. Chemical structures of folates used in the binding study

The monoglutamate and pentaglutamate forms of tetrahydrofolate are shown. In the monoglutamate form R indicates position of an H atom on the N-5 position in THF, a CH_3-group in 5-methyl-THF and a CHO-group in 5-formyl-THF.

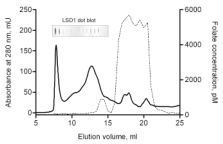

Figure 2. Folate and LSD1 in HeLa nuclear extract

The heavy 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.

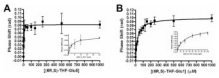

Figure 3. Representative binding curves obtained by BSI

The BSI signal is plotted vs. ligand concentration. (A) (6R,S)-THF-monoglutamate; (B) (6R,S)-THF-pentaglutamate.

Table 1

Binding of different forms of folate to LSD1. Values are the means +/− SEM of dissociation constants.

Folate	K_d μM
(6R,S)-THF-Glu5	2.77 +/− 0.46
(6R,S)-THF-Glu1	30.3 +/− 3.8
(6S)-THF-Glu1	19.5 +/− 5.0
(6S)-5-CH3-THF-Glu1	46.3 +/− 12.3
(6S)-5-CHO-THF-Glu1	72.9 +/− 16.2
(6R,S)-THF-Glu5 – Truncated LSD1	4.02 +/− 0.92
(6S)-5-CHO-THF-Glu1, no BME	65.1 +/− 14.0