Folate deficiency affects histone methylation

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Abstract

Formaldehyde is extremely toxic reacting with proteins to crosslinks peptide chains. Formaldehyde is a metabolic product in many enzymatic reactions and the question of how these enzymes are protected from the formaldehyde that is generated has largely remained unanswered. Early experiments from our laboratory showed that two liver mitochondrial enzymes, dimethylglycine dehydrogenase (DMGDH) and sarcosine dehydrogenase (SDH) catalyze oxidative demethylation reactions (sarcosine is a common name for monomethylglycine). The enzymatic products of these enzymes were the demethylated substrates and formaldehyde, produced from the removed methyl group. Both DMGDH and SDH contain FAD and both have tightly bound tetrahydrofolate (THF), a folate coenzyme. THF binds reversibly with formaldehyde to form 5,10-methylene-THF. At that time we showed that purified DMGDH, with tightly bound THF, reacted with formaldehyde generated during the reaction to form 5,10-methylene-THF. This effectively scavenged the formaldehyde to protect the enzyme.

Recently, post-translational modifications on histone tails have been shown to be responsible for epigenetic regulation of gene expression. One of these modifications is methylation of lysine residues. The first enzyme discovered to accomplish demethylation of these modified histones was histone lysine demethylase (LSD1). LSD1 specifically removes methyl groups from di- and mono-methylated lysines at position 4 of histone 3. This enzyme contained tightly bound FAD and the products of the reaction were the demethylated lysine residue and formaldehyde. The mechanism of LSD1 demethylation is analogous to the mechanism previously postulated for DMGDH, i.e. oxidation of the N-methyl bond to the methylene imine followed by hydrolysis to generate formaldehyde. This suggested that THF might also be involved in the LSD1 reaction to scavenge the formaldehyde produced.

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Conflict of interest

None of the authors have a conflict of interest.

Our hypotheses are that THF is bound to native LSD1 by analogy to DMGDH and SDH and that the bound THF serves to protect the FAD class of histone demethylases from the destructive effects of formaldehyde generation by formation of 5,10-methylene-THF. We present pilot data showing that decreased folate in livers as a result of dietary folate deficiency is associated with increased levels of methylated lysine 4 of histone 3. This can be a result of decreased LSD1 activity resulting from the decreased folate available to scavenge the formaldehyde produced at the active site caused by the folate deficiency. Because LSD1 can regulate gene expression this suggests that folate may play a more important role than simply serving as a carrier of one-carbon units and be a factor in other diseases associated with low folate.

Introduction

Post-translational modification of histone N-terminal tails has been shown to play a role in epigenetic regulation of gene expression. These modifications include acetylation, phosphorylation, ubiquitination and methylation [1]. Modification of histones increases gene expression by promoting a more open chromatin structure. Removal of the modification does the opposite. Methylation of histones is carried out on lysines by histone lysine methyltransferases (HKMTs) and protein arginine methyltransferases (PRMTs) [2]. There are groups of enzymes that can remove the methyl groups from different modified lysines and arginines [3,4]. The first enzyme shown to demethylate histone lysines was lysine demethylase 1 (LSD1), discovered by Shi et al. in 2004 [5]. It catalyzes the removal of a methyl group from histone 3 lysine 4 (H3-K4). This enzyme is highly specific for H3-K4 and uses FAD as the electron acceptor. A family of LSD1 enzymes have now been identified that use the same mechanism in which FAD is the electron acceptor [6]. A second family of histone demethylases comprises the JHDM enzymes that use a different mechanism for oxidation of the N-methyl bond and also produces formaldehyde (Fig. 1). They use the Fe(II)-α-ketoglutarate system with molecular oxygen to generate a hydroxylated carbinolamine that spontaneously produces formaldehyde [7]. Formaldehyde is toxic causing cross-linking of peptide chains, for example, in a group of amine oxidases with methylamine as a substrate [8]. However, in spite of producing formaldehyde as a product, the reason for the absence of damage to either class of histone demethylases is unknown.

Over 30 years ago we identified the mitochondrial enzymes, dimethylglycine dehydrogenase and sarcosine dehydrogenase, as having tightly bound tetrahydrofolate when purified from rat liver [9–11]. We obtained indirect evidence that THF combines with formaldehyde formed during the reaction to produce 5,10-methylene-tetrahydrofolate ($5,10$-CH_2-THF) [11]. This suggested that THF scavenges the toxic formaldehyde to protect the enzyme during the reaction. The formation of $5,10$-CH_2-THF from THF and formaldehyde is a well-known reversible chemical reaction [12]. $5,10$-CH_2-THF is a carrier of one-carbon units in the cell at the formaldehyde level of oxidation and is important in the synthesis of serine from glycine by serine hydroxymethyltransferase as well as other reactions e.g. thymidylate synthetase. The mechanism of the reactions catalyzed by DMGDH and SDH was proposed to be an initial oxidation of the N-methyl bond to an imine using bound FAD as the electron acceptor followed by hydrolysis to yield the demethylated substrate and a compound

referred to as 'active formaldehyde' [13]. 'Active formaldehyde' was later identified as 5,10-methylene-tetrahydrofolate, a member of the folate family of coenzymes.

Shi et al. suggested a mechanism for removal of the methyl group from H3K4me2 by LSD1 in their initial paper that was remarkably similar to the mechanism that we had proposed for the demethylation of dimethylglycine by DMGDH [9]. Both enzymes contain tightly bound FAD. Both produce formaldehyde as the product of the demethylated methyl groups. Both are active when measured in tissue extracts in spite of the formaldehyde produced. However, only DMGDH was known to contain bound THF. This suggested that LSD1 might also contain bound THF to complex the formaldehyde generated at the active site. Another difference between the two sets of experiments is that DMGDH was isolated from rat liver while LSD1 was a recombinant protein isolated from overexpressed *Escherichia coli*. We then showed that recombinant LSD1 bound the natural form of THF specifically and tightly [14]. Further evidence that THF bound to LSD1 could serve as a scavenger of the formaldehyde produced during the reaction was provided by a crystal structure of LSD1 complexed with THF. This showed that THF was positioned facing the isoalloxazine ring of FAD to accept to accept formaldehyde as it is generated [15].

Our first hypothesis is that LSD1, and perhaps other members of the FAD family of histone lysine demethylases, also contain bound THF in tissue. Our second hypothesis is that the bound THF protects these demethylases by formation of 5,10-methylene-THF using a mechanism similar to that operating in DMGDH and SDH to scavenge formaldehyde and protect the enzyme. Confusion regarding the role of THF in protection of DMGDH and SDH complicates our understanding of the mechanism. When tissue extracts are prepared and enzyme activity is measured, the demethylation proceeds in the absence of added THF. However, if THF is added to the reaction mixture, 5,10-methylene-THF is formed [16]. In the case of DMGDH and SDH it appears that enzyme-bound THF *in situ* dissociates when extracts are prepared for measurement of enzyme activity. Dilution of the enzyme results in loss of THF and decreased concentrations of both formaldehyde and the enzyme protein susceptible to formaldehyde cross-linking. Therefore, when concentrations of both formaldehyde and DMGDH or SDH have decreased, damage to the enzyme is minimal. This may be the reason that THF is needed to protect the enzymes *in situ* but is not needed when activity is measured in tissue extracts.

Unlike the studies that were carried out on DMGDH and SDH, all studies on LSD1 were carried out using enzyme purified by overexpression in *E. coli*. It is unlikely that an overexpressed protein will contain significant amounts of a non-covalently bound cofactor since only the protein was overexpressed and reaction mixtures probably do not contain significant amounts of THF. In spite of the generation of formaldehyde, enzymatic measurements of LSD1 activity proceed in the absence of THF. This is probably because the reaction mixtures were too dilute to permit the formaldehyde produced to react with the protein.

In support of our hypothesis we now report Pilot Data showing that the amount of dimethyl-H3K4, the LSD1 substrate, is greater in tissue from folate-deficient mouse liver than from non-deficient liver. This is consistent with a decreased amount of THF present at the active

site of LSD1, leading to diminished ability to scavenge the formaldehyde produced and decreased LSD1 activity. Measurements of dimethylH3K4 were made qualitatively by Western blotting using antibody to a synthetic LSD1 substrate and confirmed quantitatively by mass-spectrometry.

Pilot data

Materials and methods

Animals—The Animal Care Committee of Vanderbilt University School of Medicine approved animal studies. Weanling wild type male C57BL6/J mice were fed the folate-deficient diet obtained from Dyets or the same diet supplemented with 1 mg. folic acid/kg of diet as described previously [17]. Animals were euthanized at 57 days and the livers removed.

Western blotting—Histones were extracted by a commercial kit (EpiSeeker Histone Extraction Kit from Abcam). All antibodies were from Abcam. To make a correct assessment of the difference in H3K4 methylation, the blotting was normalized to total histone H3. This was done by Western blotting first with antibodies against total histone H3. Based on the result from the first blot, the samples were normalized to load the same amount of total H3 on the next Western blot. In this case antibodies against dimethylated K4 of H3 were used. Blotting was visualized by a standard chemiluminescence method.

Measurement of histone modification by mass-spectrometry—This was carried out using mass spectrometry-based proteomics developed by the Garcia lab [18]. The total histones extracted are chemically derivatized using propionic anhydride and then individual histones are digested enzymatically using trypsin, followed by one more round of propionylation. The peptide digests are then desalted using home- made STAGE tips and then the peptides are separated and identified using on-line nano liquid chromatography directly coupled to an Orbitrap Elite mass spectrometer electron transfer dissociation (ETD) mass spectrometry. The identified peptides are each separated and their post-translational modifications are then determined using MS/MS fragmentation. The peptides are quantified using the EpiProfile program where each modification is expressed as a percentage of the total modification of the particular peptide [19].

Results and discussion

Folate deficiency can be produced in mice by using a synthetic amino acid-based diet containing an antibiotic such as succinylsulphathiazole to prevent the bacterial flora from providing folate to the animals. Such animals have defective hematopoiesis [17]. Fig. 2 shows that weanling male mice fed this diet without folate began to lose weight after 20 days compared to animals fed the diet supplemented with folic acid. There were 5 animals in each group and were weighed every 2 days. After 57 days the animals fed the folate-deficient diet had lost 20% of their maximum weight and animals of both groups were euthanized. Tissues were removed and kept frozen for further analysis. Histones were extracted from the livers of 3 animals from both groups. Western blots were prepared to determine the content of the histone substrate for LSD1. LSD1 is able to remove methyl

groups from H3K4me2 and H3K4me1 but not H3K4me3. When equal amounts of histone 3 were used in the blots and then probed with antibody to H3K4me2, there was a marked difference in the amount of H3K4me2 that was present in samples from the folate-deficient animals compared to those from the folate supplemented animals (Fig. 3). The increased amount of H3K4me2 that is present in the tissue from the folate-deficient animals is consistent with decreased activity of LSD1. If there is less THF present at the active site there would be less formaldehyde converted to $5,10\text{-}CH_2\text{-}THF$. This could result in damage to the enzyme and support our hypothesis that the role of bound THF is to scavenge the toxic formaldehyde as it is produced. The results presented in Fig. 3 are necessarily qualitative. In order to confirm these observations we also measured the abundance of histone H3 marks, especially the H3K4me2 mark, by quantitative mass spectrometry (MS). The data presented in Table 1 shows the relative abundance of some post-translational modifications on histone 3 from folate-deficient and folate-supplemented mouse livers. All the unmodified and methylated states of H3K4 are derived from the parent peptide TKQTAR, (amino acids 3–8 of histone 3). The values in the Table are the relative abundance of a particular modification compared to all the other modifications derived from the parent peptide, expressed as a percent. It will be noted that the percent of H3K4me2 from the non-deficient animals is half that of the deficient animals. This is a highly statistically significant difference and is similar to the qualitative result obtained by Western blotting in Fig. 3. There is no significant change in H3K4me1 although it is also a substrate for LSD1. However, this might stem from the fact that the monomethyl form may be both increasing and decreasing at the same time. H3K4me3 is not a substrate for LSD1. It is unaffected in the MS results.

Table 1 also presents data for the unmethylated and methylated forms of H3K9. It was earlier reported that methylated H3K9 is also a substrate for LSD1 [20] although recent reports indicated mono- and dimethylated H3K9 are not substrates [21]. The fact that values for H3K9me2 are unchanged by folate deficiency, unlike the differences observed for H3K4me2, is consistent with it not being a substrate.

It might be argued that the folate-deficient animals were in very poor health. These animals have ''ineffective hematopoiesis" leading to pancytopenia and that decreased activity of LSD1 might be unrelated to a decreased ability to scavenge formaldehyde but, instead, a result of a general deterioration of cellular function. If that were so, then one would expect folate deficiency to result in decreased activity of histone deacetylase. Formaldehyde is not a product of that reaction. Table 1 shows values for three different histone 3 acetylated modifications. In no case was there a difference in the level of acetylation between folate-sufficient and folate-deficient tissues.

These results lead us to formulate the following hypotheses. First, that cellular LSD1 contains bound THF *in vivo*. Second, that the bound THF serves to scavenge formaldehyde produced during the demethylase reaction and protects LSD1 from formaldehyde damage. Evidence to support this is provided by the similarity in reaction mechanisms between LSD1 and DMGDH. The latter reaction has been shown to involve formation of $5,10\text{-}CH_2\text{-}THF$ [11]. Also, studies from our laboratory have shown that purified LSD1 binds the natural form of THF tightly and specifically [14]. In addition, a crystal structure of LSD1 obtained

with THF shows that it is bound close to FAD in position to accept formaldehyde generated by the reaction [15]. What would be the purpose of having such a specific binding site if not to be filled by THF?

What are the possible arguments against these hypotheses? First, THF has never been found to be present bound to LSD1. We believe this is because it was never needed as a reactant when enzyme activity was measured in vitro and, therefore, no one has looked for it. Second, with DMGDH and SDH, THF is not needed when these enzymes are measured in vitro. DMGDH and SDH enzyme activities are easily measured in purified extracts of liver and formaldehyde is a product of the reaction [22]. But, in spite of the fact that they contained THF, $5,10$-CH$_2$-THF was not a product of the enzymatic reaction measured in vitro unless added to the reaction mixture and the formaldehyde produced did not inactivate the enzymes. Why, then, is the enzyme still active in tissue extracts used to measure enzyme activity? It seems reasonable to assume that most of the bound THF had dissociated from the enzyme during the extraction and purification procedure. However, when THF was added, $5,10$-CH$_2$-THF was formed [16].

When measurement of recombinant LSD1 activity (using a 21-amino acid alternative substrate) was carried out in the presence of added THF, there was no effect on the rate of product formation or substrate disappearance [15]. One explanation for this is that the formation of $5,10$-CH$_2$-THF from THF and formaldehyde is not the rate-limiting step in the reaction. Another explanation is that another factor may be needed for THF binding. In the cell, LSD1 demethylates histone H3 as part of a multimeric protein complex in which it directly interacts with the co-repressor of the repressor element 1 silencing transcription factor, CoREST. Interaction of these two proteins has been studied in detail [23]. The LSD1–CoREST complex maybe needed for THF to bind under in vitro conditions. The crystal structure showing THF bound at the active site was carried out using the LSD1–CoREST complex [15] .

There are two classes of histone demethylases. They both use the same general strategy to break the thermodynamically robust N-methyl bond. The methyl-amino group is first oxidized to a methylimine followed by hydrolysis to produce formaldehyde (Fig. 1). The two classes differ in the mechanism of oxidation. In the FAD class, tightly bound FAD serves as an electron acceptor and the reduced FAD is reoxidized with the formation of H_2O_2. The other class, termed the JHDM (also called the Jumonji) class, uses molecular O_2, Fe^{++} and α-ketoglutarate to oxidize the N-methyl bond. Both classes produce formaldehyde as a product.

The mechanism of the demethylase reactions carried out by DMGDH and SDH is very similar to that employed by LSD1, suggesting that THF may also play a role in protecting LSD1 from damage. But when the reaction was carried out in vitro using a 21 amino acid fragment of histone H3 that is a substrate for LSD1 the reaction proceeded in the absence of THF [15].

This paradox may be explained in the following way. Although THF is not needed for lysine demethylation to proceed in diluted tissue extracts it might be needed in order to protect the

enzyme *in vivo* when it is bound at the active site. In order to test this it would be necessary to remove THF from its binding site in the native enzyme *without* making a tissue extract. It is possible to do so by using tissue from folate-deficient animals. This will decrease the amount of all folate cofactors in livers of these animals and the amount of THF bound to the enzyme *in situ*. If the purpose of the bound THF, indeed, *were* to scavenge formaldehyde and protect the enzyme, then in the absence of THF the enzyme would not be protected and the activity of the enzyme would be decreased. This is what we have shown qualitatively in Fig. 3 and quantitatively in Table 1. The fact that LSD1 (also known as KDM1) regulates the epigenetic expression of many enzymes and metabolic processes indicates that folate deficiency may have a much greater metabolic role than simply serving as a carrier of one-carbon units [24].

Folate deficiency has been linked to a number of human conditions. Neural tube defects are the most outstanding example. This has led to the fortification of the diet with folic acid in the United States and several other countries with remarkable success in reducing the incidence of births with neural tube defects. Yet there is no known specific mechanism for how folate is involved in the formation of the neural tube in the early development of the embryo. Other studies have linked folate deficiency to autism [25], Alzheimer's disease [26] and senile dementia [27] with no evidence for how this can take place. If LSD1 can use THF to scavenge formaldehyde to protect the enzyme from damage and if folate deficiency results in decreased LSD1 activity, LSD1 regulation of gene expression may be affected. Studies using specific inhibitors of LSD1 have indicated that many different biological systems are impaired [28]. Our data suggest that folate deficiency can act similar to chemical inhibitors of LSD1 and affect many enzyme systems.

In conclusion, we believe that the activity of the folate coenzymes is not limited to carrying one-carbon units in metabolic pathways but may have an epigenetic role as well in diseases that have been shown to be associated with low folate.

Acknowledgements

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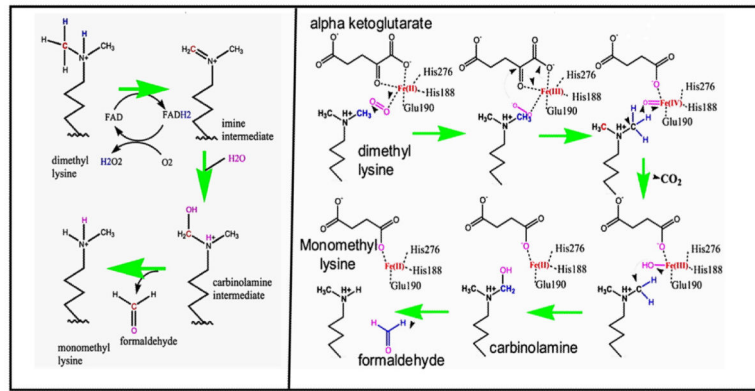
Fig. 1.
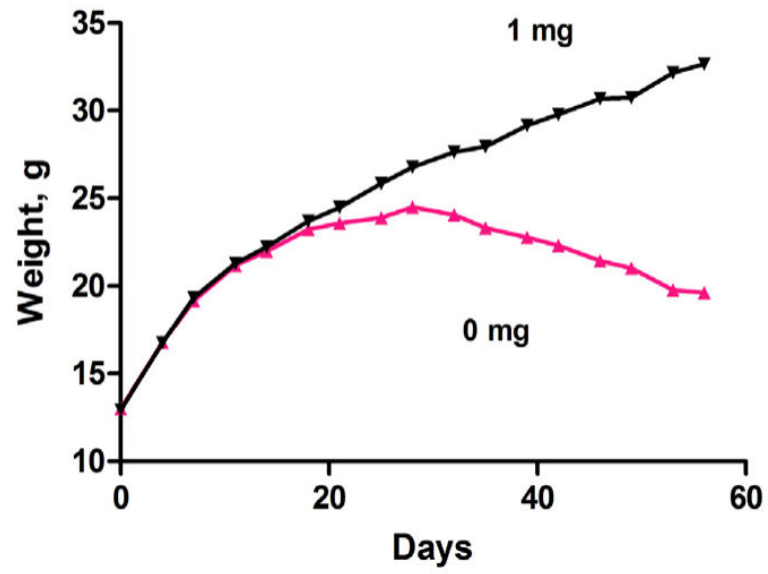FAD class on left. Alpha ketoglutarate-Fe^{++} class on right. From Ref. [7].

Fig. 2.
Weight gain of the mice on normal and folate-deficient diet. C57BL6/J mice used.
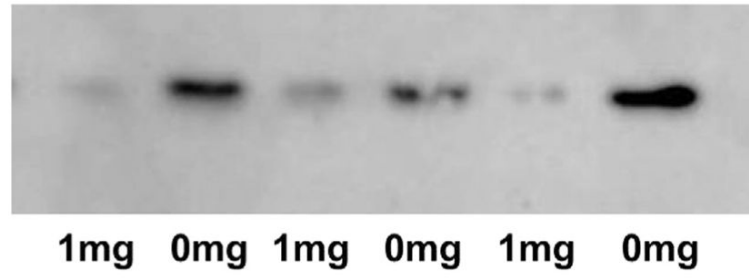
Fig. 3.
Western blotting of mouse liver histone extracts with antibodies against H3-dimethyl-K4. 1 mg, C57BL6/J mouse fed normal diet (1 mg folate/kg diet); 0 mg, mouse fed folate-deficient diet (0 mg folate/kg diet).

Table 1

Histone Modifications.

Parent peptide	Histone modification	Minus folate	Plus folate	Difference	*P* value
TKQTAR(H3 3-8)	Unmodified	90.6 ± 0.49	90.8 ± 1.11	0.22 ± 1.9	0.86 ns
	M3K4me1	8.55 ± 5.44	8.61 ± 8.99	0.06 ± 1.1	0.95 ns
	H3K4me2	0.29 ± 0.04	0.14 ± 0.03	0.15 ± 0.1	**0.03 S**
	H3K4me3	0.09 ± 0.02	0.24 ± 0.08	0.16 ± 0.1	0.13 ns
KSTGGKAPR(H3 9-17)	Unmodified	0.14 ± 0.01	0.12 ± 0.03	0.02 ± 0.03	0.50 ns
	H3K9me1	0.06 ± 0.01	0.06 ± 0.02	0.00 ± 0.02	0.86 ns
	H3K9me2	0.30 ± 0.02	0.33 ± 0.02	0.03 ± 0.03	0.26 ns
	H3K9me3	0.17 ± 0.01	0.17 ± 0.01	0.00 ± 0.01	0.94 ns
	H3K14ac	0.08 ± 0.01	0.06 ± 0.01	0.02 ± 0.01	0.16 ns
KQLATKAAR(H3 18-26)	Unmodified	0.71 ± 0.01	0.69 ± 0.03	0.02 ± 0.03	0.44 ns
	H3K18ac	0.02 ± 0.00	0.03 ± 0.00	0.00 ± 0.01	0.56 ns
	H3K23ac	0.21 ± 0.01	0.21 ± 0.02	0.01 ± 0.02	0.76 ns

The first column shows the parent peptides that are obtained by proteolytic digestion of histone 3. The amino acid number of the peptide is in parenthesis. The second column shows the specific histone modification that is derived from the parent peptide by nanoLC–MS/MS. The values in columns 3–5 are the relative abundance of the specific modification in the parent peptide expressed as a % of all the modifications derived from the parent peptide. They are the means (±SEM) of separate analyses of samples from histone 3 from livers of three animals grown in the absence or in the presence of folate. The data were analyzed using Prism employing an unpaired two tailed *t* test.