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LSD1 and The Chemistry of Histone Demethylation

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Abstract

The recent discovery that histone demethylation can be catalyzed by the flavin-dependent amine oxidase LSD1 has ushered in a new chapter in the chromatin remodeling community. Herein we discuss the rapid progress of the histone demethylase field including: the recent identification of the nonheme iron-dependent histone demethylases (JmjC family), the basis for LSD1 substrate site-specificity, and the newly emerging potential for inhibition of these enzymes in structural and functional analysis.

Introduction

One of the surprises in determining the sequence of the human genome was how relatively few genes (ca. 25,000) are encoded by our DNA [1]. As a result, biologists have come to appreciate more than ever that the informational diversity that leads to our complexity stems from post-transcriptional mechanisms including RNA splicing and protein post-translational modifications. The latter allows for a rich variety of covalent protein structural changes that includes phosphorylation, acetylation, ubiquitylation, sulfation, glycosylation, lipidation, and methylation [2]. These changes in protein chemical structure often result in profound influences on the mechanism and function of proteins in cells.

Core histone proteins are highly conserved and play a critical role in modulating chromatin structure and DNA accessibility for replication, repair, and transcription [3]. There has been intense interest in mapping and characterizing the histone post-translational modifications that guide chromatin remodeling and account for epigenetics. In this regard, the protein lysine modifications have a special place in the history of gene regulation. Allfrey and colleagues demonstrated in the 1960's that histone lysine acetylation was often found in chromatin that was in an activated state [4]. Histone lysine methylation was proposed to be reciprocal with acetylation in structure and function. We now know that the effects of lysine acetylation and methylation are more variegated than these early concepts and depend on specific modification sites as well as additional factors at specific genes. The progress of research in this field has been catapulted by a series of discoveries of key enzymes that catalyze histone lysine acetylation and deacetylation [5,6] on the one hand and methylation and demethylation [7, 8•] on the other.

The first specific histone lysine methyltransferases were reported in 2000 and as expected were S-adenosyl methionine dependent enzymes [7]. There are at least two classes of histone lysine methyltransferases, the SET domain containing protein family [9] and the non-SET domain protein analogues of DOT1 [10-12]. Several of these methyltransferases are quite specific for

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their targeted lysine and preferentially add one, two or three methyl groups. For many years, it was uncertain if methylation marks on histones were “permanent”. Unlike the reversibility of most post-translational modifications, which involve hydrolytic reactions, it seemed less likely that lysine methylation would be cleaved in this manner because of the kinetic stability of the N-Me bond. In 1973 Paik and colleagues demonstrated the enzymatic demethylation of N-methylated calf thymus histones, although they were unable to ultimately identify the corresponding enzyme [13].

Lysine Demethylases

In late 2004, it was reported that a flavin dependent amine oxidase that appeared to be a polyamine oxidase homolog, LSD1, was capable of selective demethylation Lys-4 of histone H3 [8••]. This study laid to rest the debate as to whether or not lysine methylation was a static epigenetic mark and opened up a new chapter in the chromatin field. Since the initial description of LSD1, there has been a frenzied pace of molecular biology discovery in reversible protein methylation. At least ten other lysine demethylases, belonging to the Jumonji C or JmjC family of iron(II)-alpha-ketoglutarate dependent demethylases have been described in the literature, with varying specificities for lysine site and the degree of methylation of the substrate [14-24] (Table 1). To some extent, the biochemical analysis of these JmjC proteins has lagged behind their genetic roles. Of some concern, there have been few kinetic studies reported for the JmjC catalyzed reactions, and estimated values deduced from published data suggest that they may have turnover numbers in the range of 0.01 min^{-1} with histone substrates, about 300-fold lower than LSD1 [25]. These unusually slow reaction rates may simply reflect the need for yet to be identified accessory proteins to stimulate JmjC activities but leave open the possibility that alternative substrates exist.

In contrast, the biochemical studies on LSD1 have moved at a somewhat brisker pace and leave little doubt at this juncture that LSD1 is a bona fide histone demethylase. LSD1's architecture contains a classical amine oxidase catalytic domain, an N-terminal SWIRM domain, which has been proposed to be involved in nucleosomal targeting [26], and an N-terminal extension [8••]. The catalytic turnover of LSD1 proceeds as expected for an FAD-dependent amine oxidase. Concurrent amine oxidation and flavin reduction are followed by the re-oxidation of flavin by one equivalent of molecular oxygen, producing stoichiometric hydrogen peroxide along with formaldehyde as the demethylation byproduct (Figure 1a). Since both hydrogen peroxide and formaldehyde have mutagenic potential and LSD1 is producing them in the nucleus, the stakes for detoxification pathways and tight control of enzymatic activity must be considerable. It has been proposed that the byproduct formaldehyde can be formally recycled and ultimately become the donated methyl of S-adenosyl methionine dependent histone methyltransferases [27].

LSD1 Reaction Mechanism

LSD1 is a member of the monoamine oxidase family based on sequence and recent X-ray crystal structures [8••,28] and there has been a great deal of research into the detailed catalytic mechanisms of these enzymes. The FAD cofactor is oxidized by molecular oxygen, presumably by a single electron mechanism. Studies with LSD1 suggest that this flavin oxidation is rapid [29], based on changes in the flavin optical spectrum which show classic FAD absorbance maxima at approximately 460 and 380 nm in the resting state, assigned to fully oxidized flavin and a one electron reduced semiquinone form [30]. The precise mechanism of the oxidative conversion of the amine to an imine is controversial and may involve either hydride transfer or single electron mechanisms [31,32]. Elegant recent studies on the flavin-dependent oxidase tryptophan 2-monooxygenase may shed light on the general reaction class. By measuring deuterium and ^{15}N isotope effects on the amine to imine reaction, the authors found a large

deuterium effect (6.0 ± 0.5) and an inverse ^{15}N isotope effect on CH bond cleavage (0.9917 ± 0.0006) [31]. This was interpreted in favor of a hydride transfer mechanism rather than a radical mechanism for amine oxidation since such a large deuterium effect is consistent with irreversible CH bond cleavage. Whether this will hold for all other related amine oxidases will require further investigation. Following imine formation, hydration to the N,O-hemiacetal and subsequent collapse to formaldehyde and amine are presumed to be relatively rapid and spontaneous reactions. It is clear that the trimethylammonium functionality could not be a substrate for the flavin amine oxidases because of the absence of a nitrogen lone pair, which is integral to the reaction. In contrast, the iron(II) α -ketoglutarate oxygenases can hydroxylate alkyl groups directly through a hydroxyl radical, providing a chemically plausible path to demethylation of quaternary ammonium salts (Figure 1b).

LSD1 Substrate Specificity

LSD1 is most closely related to the polyamine oxidase superfamily and LSD1 was originally tested with various small molecule polyamines as potential substrates, but these compounds showed minimal or undetectable turnover under the reaction conditions [8•]. Various methyl-lysine peptides derived from histones were then investigated as possible substrates, including mono- di- and tri-methylated Lys-4 H3 tail peptides, full-length Lys-4-methylated histone H3, and dimethyl-Lys-9, 36, and 79 of H3 and Lys-20 of H4 containing peptides [8•]. A high level of substrate specificity for Lys-4 of H3 was suggested since no other methyl-lysine sites were processed during this *in vitro* analysis [8•]. Forneris *et al.* demonstrated that histone H3 tail peptides greater than 16 amino acids in length are necessary to achieve high demethylase efficiency, furthermore LSD1 does not have a strong kinetic preference for the mono- or dimethyl forms of Lys-4 based on histone H3 synthetic tail peptides [33•]. Epigenetic post-translational modifications of Lys-9 (methylation, acetylation), Ser-10 (phosphorylation), Lys-14 (acetylation), Lys-18 (acetylation), Arg-2 (methylation), Arg-8 (methylation), and Arg-17 (methylation) of histone H3 and their influence on LSD1 Lys-4 demethylation of an H3 tail peptide have been explored [29,33•]. Interestingly, nearly all of these modifications led to a drop in enzymatic demethylation efficiency by LSD1, revealing the high specificity for the native histone tail sequence and the potential importance for electrostatic interactions between histone and LSD1. Consistent with the important role for electrostatic interactions, demethylation rate is very sensitive to ionic strength [33•]. This selectivity correlates nicely with the known function of LSD1 as part of a general transcriptional repressor complex along with CoREST and HDAC1/2 [34-35]. Since methylation of Lys-4 is a mark of gene activation, methyl removal at this site is gene silencing. Interestingly, there are reports that the LSD1 substrate specificity can be modulated *in vivo* by protein-protein interactions [36,37], but this has yet to be confirmed *in vitro*.

Structural Studies on LSD1 in Complex with a Histone Analog Suicide Inhibitor

Insertion of various functional groups including cyclopropyls and propargyls into monoamine oxidase substrates convert these compounds into mechanism-based inactivators [38,39]. Substitutions at the epsilon position of Lys-4 of histone H3 peptides including propargylamine, aziridine, and cyclopropylamine have been synthesized and the propargylamine containing peptide behaves as a classical mechanism-based inactivator [40•]. Based on a combination of mass spectrometry and optical and NMR spectroscopy, an inactivation mechanism in which the propargylamine is initially oxidized to the conjugated imine which undergoes Michael addition with the flavin cofactor has been proposed [24,40•] (Figure 2a). The resultant N^5 flavin adduct generated leads to a stably bound enzyme-inhibitor complex. Taking advantage of this behavior, a recent 2.7 Å crystal structure was obtained of LSD1 after treatment with a N-methylpropargylamine analog induced inactivation [41•]. In order to see electron density for

the peptide moiety, it was necessary to treat the inactivated complex with sodium borohydride prior to crystallization, which presumably led to reduction of the linker double bonds between the flavin and peptide inactivator (Figure 2b). The importance of borohydride treatment was rationalized as increasing the resistance of the adduct to hydrolysis as well as eliminating the potential heterogeneity of the double bond stereoisomers associated with the parent adduct.

The structure of the LSD1-inactivator complex, obtained in the presence of CoREST, was noteworthy in several respects. First, only residues 1-7 of the histone tail peptide gave sufficiently strong electron density to be modeled (Figure 3a). Weak density for H3 residues 8-21 may have been detected extending toward the SWIRM domain but it was insufficient to accurately model. The importance of residues beyond 7 for demethylase action is established [33•] so it is likely that the LSD1-inactivator structure is incomplete in detailing biologically important recognition. Nevertheless, the H3 residues visualized formed a very unusual series of three gamma turns in the secondary structure [41•] (Figure 3b). Gamma turn structure involves backbone hydrogen bond interactions between the *i* and *i*+2 residues in a protein chain providing a pseudo 7-membered ring [42] (Figure 3c). Based on related structural studies, most enzymes involved in post-translational modifications recognize extended conformations of peptide substrates proximal to the targeted residue. To our knowledge, this interaction between LSD1 and the tail of H3 is unique among characterized enzyme-peptide substrate complexes.

Given this unusual conformation observed in the LSD1 complex, it is appropriate to question its biological relevance, especially since it was obtained with a suicide inactivator. However, the crystal structure reveals a series of hydrogen bond and van der Waals interactions between the side chains of H3 residues and those of LSD1, effectively predicting the behavior of enzyme mutants and alternative substrates [41•]. For example, the LSD1 structure shows that the free N-terminal amino group of H3 makes a key electrostatic interaction with Asp-555 of LSD1. Replacement of the amino group by a methyl group, charge masking by an acetyl group, or mutagenesis of Asp-555 all result in a major loss in substrate processing. Analogous experiments confirm the key interactions between Arg-2 of histone H3 and LSD1 residues Asp-556 and Trp-552. Overall, the LSD1 inactivated structure reveals an elegant basis for the highly specific demethylation for Lys-4 of histone H3. The ability of amino terminal acetylation to reduce processing efficiency by LSD1 may have biological significance for interpretation of the histone code since histone N-terminal acetylation is likely to occur reversibly *in vivo*.

In Vivo and Nucleosomal Targeting

Relatively little is known about the structural basis of demethylation of chromatin by LSD1 *in vivo*. However, it appears that CoREST plays an important role in targeting histone H3 demethylation in the context of nucleosomes [34,35]. Knockdown of CoREST greatly reduces the efficiency of LSD1-mediated histone demethylation *in vivo* and biochemical studies with nucleosome substrates have confirmed this behavior. Based on NMR and mutagenesis experiments it has been proposed that CoREST may target LSD1 to DNA [28].

In separate experiments, it has been proposed that LSD1 can demethylate Lys-9 of histone H3 in different physiologic contexts [36,37]. In this model, androgen receptor interactions with LSD1 are thought to dictate this substrate specificity change. If correct, these data suggest that LSD1 can have a gene activation function as well as silencing effect, depending on promoter context. These data have not yet been supported by *in vitro* biochemical studies, nor does the LSD1-H3 crystal structure provide a clue as to how this could be accomplished [41•]. However, recent reports that the *S. pombe* homologues of LSD1, *splsd1* and *splsd2*, may target Lys-9 for demethylation adds plausibility to the altered substrate specificity in mammalian systems [43].

LSD1 Small Molecule Inhibitors

LSD1 as a therapeutic target for small molecule inhibitors is plausible in analogy to the now clinically validated epigenetic HDAC enzyme class. Along these lines, the cyclopropylamine containing monoamine oxidase inhibitor tranylcypromine was proposed to inhibit LSD1 [44•] and further characterized as a mechanism-based inactivator of LSD1 [45,46]. It is noteworthy that a cyclopropylamine substrate analog of histone H3 failed to induce inactivation of LSD1, suggesting tranylcypromine adopts a distinctive set of enzyme interactions [25]. The recent kinetic and structural studies have investigated the mechanism of inactivation by tranylcypromine; the authors of both studies propose similar mechanisms to that of Figure 2c. While less potent against LSD1 than against monoamine oxidase B, tranylcypromine thus appears to represent a promising lead agent for further development. Recently, a series of bisguanidine and biguanide polyamine analogs were tested for inhibitory activity against LSD1. The *in vitro* analysis characterized these compounds as being noncompetitive in nature [47]. Interestingly, these compounds proved successful in reactivating the expression of silenced genes associated with tumor suppression, hinting at the therapeutic potential of LSD1 inhibition in the treatment of cancer [47].

Conclusion and Future Challenges

It is difficult to overstate the briskness of research in the histone demethylation field set-off by the initial report of LSD1 less than three years ago [8••]. The flavin- and iron-dependent demethylases appear to play key roles in gene regulation and cellular growth and differentiation. Still, there are many remaining challenges. How precisely do these demethylases catalyze reactions and recognize their substrates and how does the *in vivo* context modify and regulate these interactions? Recently, several crystal structures of the JmJc containing protein JMJD2A bound to substrate analogs have been reported and these should help clarify the basis for specificity and catalytic action [48-50]. Also, a second structure of the LSD1-CoREST complex with a substrate mimic has been shown, introducing the possibility of multiple substrate binding conformations [51]. An increased effort on structural and enzymatic studies which take into account partner proteins and nucleosomal assemblies will be needed. Will there be therapeutic potential in the recognition of small molecule gamma turn mimics or will other small molecule scaffolds offer greater promise? Here the synthetic chemist will have an important role to play. Are there other flavin-dependent protein demethylases lurking in the mammalian genome and are there bona fide protein substrates outside of histones? Chemical proteomic approaches involving mechanism-based inactivators may play a useful role in this regard [25]. How do the redox chemistries of the flavin- and iron-dependent demethylases affect the cell nucleus and what protective measures are most critical in preventing DNA damage? There has been a growing interest in the role that environmental-toxin induced oxidative damage can play in disease progression, and it would seem that attention to endogenous sources of chemically reactive species should also be considered. Are there bona fide CpG island DNA demethylases? The chemical challenge of this class of reaction is even more difficult than for protein demethylation, but it remains possible that such DNA-targeted enzymes have yet to be uncovered. We look forward to the years ahead and the likelihood of many more exciting discoveries and developments in the oxidative demethylation field which will rely heavily on the involvement and ingenuity of chemical biologists.

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Figure 1. Catalytic mechanisms of demethylase enzymes

(a) The FAD dependent demethylation of Lys-4 of histone H3 proceeds through the hydrolysis of an iminium ion following a two electron oxidation of the amine by the flavin. R = ribosyl adenine dinucleotide **(b)** The iron(II) dependent demethylation of trimethyl-lysine substrates proceeds through an iron(II), α -ketoglutarate, and O_2 derived hydroxyl radical oxidation of the methyl C-H bond.

Figure 2. Mechanism-based inactivation of LSD1

(a) The proposed mechanism of inactivation of LSD1 by an N-methylpropargylamine containing H3 peptide proceeds through conjugate addition of the flavin N⁵ to the gamma carbon of the electrophile following a two electron oxidation to the iminium ion. (b) Reduction of the trimethine linkage of the FADinactivator conjugate with NaBH₄ was necessary for crystallographic studies. (c) The proposed catalytic mechanism of inactivation of LSD1 by tranylcypromine proceeds through radical recombination and subsequent dehydration following a one electron oxidation and ring opening on the amine. R = ribosyl adenine dinucleotide in a-c

Figure 3. LSD1 substrate specificity revealed by an LSD1-inactivator crystal structure

(a) Active site view of the key interactions between Asp-556 and Ala-1, the N-terminus of the H3 peptide, and both Trp-552 and Asp-556 with Arg-2 of the H3 peptide. **(b)** Active site view of the configuration of the three consecutive gamma turns that the H3 peptide backbone adopts between Arg-2 and Lys-4, Thr-3 and Gln-5, and Lys-4 and Thr-6. **(c)** Rendering of a H3 peptide containing three consecutive gamma turns to illustrate the pseudo 7-membered rings inferred by the $i, i+2$ backbone hydrogen bonding.

Table 1

Identified histone lysine demethylases.

Demethylase	Alternate Name	Site Specificity	Demethylates	Refs
Flavin dependent				
LSD1	BHC110, AOF2	H3K4	mono, di	[8••]
Fe(II) α-ketoglutarate dependent				
JHDM1A	FBXL11	H3K36	mono, di	[14]
JMJD1A	JHDM2A, TSGA	H3K9	mono, di	[15]
JMJD2A	JHDM3A	H3K9, H3K36	tri, di	[16,17]
JMJD2B	KIAA0876	H3K9	tri, di	[18]
JMJD2C	GASC1	H3K9, H3K36	tri, di	[19,20]
JMJD2D	KIAA0780	H3K9	tri, di, mono	[20]
JARID1A	RBP2	H3K4	tri, di	[21]
JARID1B	PLU-1	H3K4	tri, di	[22]
JARID1C	SMCX	H3K4	tri, di	[23]
JARID1D	SMCY	H3K4	tri, di	[24]