THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 50, pp. 41360–41365, December 16, 2005

Human Histone Demethylase LSD1 Reads the Histone Code*

Received for publication, August 30, 2005, and in revised form, October 3, 2005 Published, JBC Papers in Press, October 13, 2005, DOI 10.1074/jbc.M509549200

Federico Forneris[‡1], **Claudia Binda**[‡1,2], **Maria Antonietta Vanoni**[§], **Elena Battaglioli**[¶3], **and Andrea Mattevi**[‡4]

From the [‡]*Dipartimento di Genetica e Microbiologia, Università di Pavia, Via Ferrata 1, Pavia 27100, Italy,* [§]*Dipartimento di Scienze Biomolecolari e Biotecnologie, Università di Milano, Via Celoria 26, Milano 20133, Italy, and* [¶]*Dipartimento di Biologia e Genetica per le Scienze Mediche, Università di Milano, via Viotti 3/5, Milano 20133, Italy*

Human histone demethylase LSD1 is a flavin-dependent amine oxidase that catalyzes the specific removal of methyl groups from mono- and dimethylated Lys[4] of histone H3. The N-terminal tail of H3 is subject to various covalent modifications, and a fundamental question in LSD1 biology is how these epigenetic marks affect the demethylase activity. We show that LSD1 does not have a strong preference for mono- or dimethylated Lys[4] of H3. Substrate recognition is not confined to the residues neighboring Lys[4], but it requires a sufficiently long peptide segment consisting of the N-terminal 20 amino acids of H3. Electrostatic interactions are an important factor in protein-substrate recognition, as indicated by the high sensitivity of K_m to ionic strength. We have probed LSD1 for its ability to demethylate Lys[4] in presence of a second modification on the same peptide substrate. Methylation of Lys[9] does not affect enzyme catalysis. Conversely, Lys[9] acetylation causes an almost 6-fold increase in the K_m value, whereas phosphorylation of Ser[10] totally abolishes activity. LSD1 is inhibited by a demethylated peptide with an inhibition constant of 1.8 μM, suggesting that LSD1 can bind to H3 independently of Lys[4] methylation. LSD1 is a chromatin-modifying enzyme, which is able to read different epigenetic marks on the histone N-terminal tail and can serve as a docking module for the stabilization of the associated corepressor complex(es) on chromatin.

Histones play a fundamental role in the control of a variety of cellular processes, including gene expression, DNA replication, and repair. Histone function is modulated through covalent modifications by acetylation, methylation, ubiquitination, and sumoylation (1, 2). These modifications have specific effects and act in a combinatorial manner defining the so-called histone code (1, 3). Very recently, Shi *et al.* (4) and our group (5) have reported on the discovery of the first enzyme able to specifically demethylate Lys[4] of histone H3. The protein was therefore named LSD1 (for lysine-specific demethylase; it is also known as KIAA0601 and BHC110). The existence of histone-demethylating enzymes has always been questioned (6, 7), and with the discovery of LSD1, it was firmly established that histone methylation is a dynamic process under enzymatic control similar to the other known post-translational histone modifications. LSD1 has been typically found in association with CoREST and HDAC1/2 proteins, forming a module found in several multiprotein co-repressor complexes (8–11). Knock-out of LSD1 by RNA interference was shown to cause increased levels of histone methylation, resulting in the reactivation of a specific target gene (4). It has also been shown that LSD1 is a *bona fide* co-repressor able to repress a reporter gene and that this function is dependent on the demethylase activity (4). Based on sequence homology analysis, it was predicted that several histone demethylases are likely to exist in mammalian and other eukaryotic organisms (4).

From a biochemical and structural standpoint, LSD1 belongs to the class of flavin-dependent amine oxidases, which typically catalyze the oxidation of an amine-containing substrate using molecular oxygen as the electron acceptor (5, 12). Indeed, LSD1 catalyzes the demethylase reaction through an oxidative process (5) (Fig. 1*a*). The amino group of the methylated Lys is oxidized presumably to generate the corresponding imine compound, which is subsequently hydrolyzed to produce formaldehyde. Substrate oxidation leads to the two-electron reduction of the protein-bound FAD cofactor, which is regenerated to its oxidized form by molecular oxygen to produce hydrogen peroxide.

LSD1 catalyzes the specific removal of methyl groups from mono- and dimethylated Lys[4] of histone H3 (H3-K4)[5] (4, 5), although an androgen receptor-controlled activity on H3-K9 has also been reported (13). The histone H3 N-terminal tail is a region characterized by extreme density of covalent modifications with diverse biological meanings (reviewed in Refs. 1, 2, and 6). Among them, of special significance are positions Lys[4], Lys[9], and Ser[10], which are the most extensively and widely studied epigenetic marks of H3. Methylation of Lys[4] is generally known to activate transcription, and, therefore, the demethylase activity of LSD1 removes an activation mark. Lys[9] can be either acetylated or methylated, resulting in opposite effects; acetylation promotes formation of euchromatin, whereas methylation leads to repression of transcription. Phosphorylation of Ser[10] is a pivotal activating signal and prevents the recruitment of the transcriptional repressors by methylated Lys[9]. A key problem is now to define the biological properties of LSD1, especially in relation to its ability to "read and interpret" these epigenetic marks on H3. We addressed this problem through an *in vitro* study that probed human LSD1 (Fig. 2*a*) for its ability to act on histone peptides bearing different covalent modifications.

EXPERIMENTAL PROCEDURES

Protein Purification—Escherichia coli cells overproducing a truncated form of LSD1 lacking the N-terminal 184 amino acids (Δ184) were grown as described (5). The recombinant protein was purified following the protocol of Forneris *et al.* (5), modified by omitting the cation exchange chromatography step. The purity of the protein was monitored by SDS-PAGE (Fig. 2*a*) and UV-visible absorption spectroscopy.

* This work was supported by Ministero Dell' Istruzione, Dell' Università e Della Ricerca Grants COFIN04, FIRB01, and FIRB03, the Italian Association for Cancer Research, and Fondazione Cariplo. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] These two authors contributed equally to this work.

[2] Supported by a Young Investigator Fellowship from "Collegio Ghislieri," Pavia.

[3] To whom correspondence may be addressed. Tel.: 39-02-50315845; Fax: 39-02-50315864; E-mail: elena.battaglioli@unimi.it.

[4] To whom correspondence may be addressed. Tel.: 39-0382-985534; Fax: 39-0382-528496; E-mail: mattevi@ipvgen.unipv.it.

[5] The abbreviations used are: H3-K4, Lys[4] of histone 3; H3, histone 3; Δ184, human LSD1 mutant harboring a deletion of the N-terminal 184 amino acids; Mes, 2-(*N*-morpholinoethanesulfonic acid; Taps, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulfonic acid.

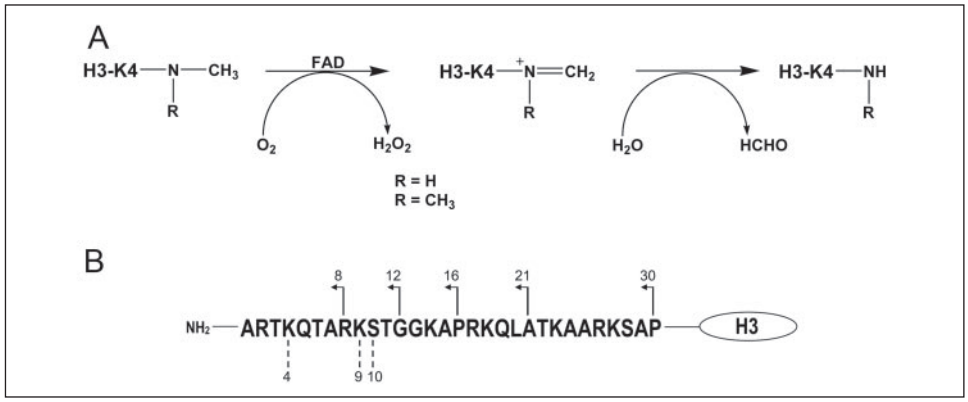

FIGURE 1. *A*, Scheme of the demethylation reaction catalyzed by LSD1. *B*, sequence of the N-terminal 30 amino acids of human H3. Lys[4], Lys[9], and Ser[10] are highlighted. The *arrows* indicate the length of the various peptides used in this work.

The recombinant protein carries an N-terminal His_6 tag to facilitate purification. Control experiments have shown that removal of the His_6 tag by proteolysis (using TEV protease) did not alter the enzyme activity by more than 5%. Protein aliquots were stored in 50 mM sodium phosphate buffer, pH 7.5, and 50% (w/v) glycerol at −80 °C. Protein concentrations were routinely measured by absorption spectroscopy using an extinction coefficient of 10,790 M^{-1} cm^{-1} at 458 nm, which was determined based on absorbance changes observed after protein denaturation in 50 mM sodium phosphate buffer, pH 7.5, plus 0.3% (w/v) SDS (14).

Activity Assays—Peptides were purchased from Thermo Electron Corp. Their purity was greater than 90% as checked by analytical high pressure liquid chromatography and mass spectrometry. All other chemicals were from Sigma. Initial velocity measurements were performed using a peroxidase-coupled assay, which monitors hydrogen peroxide production (5). The time courses of the reaction were measured under aerobic conditions by using a Cary 100 UV-visible spectrophotometer equipped with thermostated cell holder ($T = 25$ °C). Reactions were started by adding 2 μl of protein solution (40 μM protein in 50 mM sodium phosphate buffer, pH 7.5, and 5% (w/v) glycerol) to reaction mixtures (150 μl) consisting of 50 mM Hepes/NaOH buffer, pH 7.5, 0.1 mM 4-aminoantipyrine, 1 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 0.35 μM horseradish peroxidase, and variable concentrations (2–100 μM) of methylated H3-K4 peptide. Absorbance changes were monitored at 515 nm, and an extinction coefficient of 26,000 M^{-1} cm^{-1} was used to calculate the initial velocity values. After visual inspection of double reciprocal plots, which were linear, the initial velocity values, expressed as apparent turnover values, were directly fitted to the Michaelis-Menten equation (Fig. 2*b*) using Grafit (Erithacus Software), which provides the values of apparent k_{cat} and K_m along with their associated errors.

Effect of Ionic Strength and pH on LSD1 Activity—To evaluate the effects of ionic strength on LSD1 activity, assays were performed by using increasing amounts of NaCl and KCl in the reaction mixture, starting from 1 mM up to 500 mM. In the determination of the pH dependence of steady-state parameters, the pH range was covered using Mes/NaOH (pH 6.5–7.0), Hepes/NaOH (7.0–8.0), Taps/NaOH (8.0–9.0) buffers at a final concentration of 50 mM. The ionic strength was kept constant at 48 mM by the addition of an appropriate amount of NaCl (up to 32 mM). We checked that the extinction coefficient of the chromophore generated by the reaction did not change as a function of pH. The k_{cat} and k_{cat}/K_m data were best fit to the following equation, which assumes that a given parameter (P) decreases at low and high pH to a 0 value as groups with pK_{a1} and pK_{a2} dissociate (15): $\log P = \log P_{max} - \log(1 + 10^{(pK_{a1} - pH)} + 10^{(pK_{a1} + pK_{a2} - 2pH)})$.

Inhibition Studies—LSD1 inhibitors were tested by using the peroxidase-coupled assay in the presence of varied concentrations (2–100 μM) of methylated H3-K4 peptides and of the inhibitor under analysis (global range 1–300 μM, depending on the inhibitor strength). Initial velocity values were fit to equations describing competitive, uncompetitive, and noncompetitive inhibition patterns using Grafit. In all cases, the best fit was obtained with the equation describing a competitive inhibition, which allowed us to apply in some instances the Dixon plot for a graphical estimate of the K_i values. We have attempted to measure peptide binding affinities also by isothermal titration microcalorimetry; however, we found that upon the addition of the peptides, the protein solution became transiently turbid, and this feature made the usage of microcalorimetry unfeasible.

RESULTS

Activities with Monomethylated and Dimethylated 21-Amino Acid Peptides—Biochemical analyses were performed using a recombinant N-terminally truncated form of human LSD1 (5) lacking the first 184 amino acids (we refer to the sequence deposited in the NCBI data base with accession code BAA25527; 886 amino acids). This protein form comprises the SWIRM and polyamine oxidase domains of LSD1 (9); analysis of the amino acid sequence by bioinformatic tools predicts that the 184 truncated amino acids do not fold into an ordered well defined conformation (5). Furthermore, control experiments (not shown) demonstrated that the catalytic properties of Δ184 are indistinguishable from those of longer variants lacking the first 157 and 34 residues, respectively, the latter being the protein variant used by Shi *et al.* (4). The Δ184 mutant was chosen, because it can be overproduced in *E. coli* cells and readily purified to a stable and homogeneous form in the amounts required to perform the described experiments (Fig. 2*a*). LSD1 activity was monitored using histone peptides, which allowed us to quantitatively evaluate the binding and catalytic parameters and the differential effects of various covalent modifications.

The first question we addressed in our study was the difference (if any) in the catalytic efficiency toward mono- and dimethylated H3-K4 substrates. We performed steady-state kinetic analysis using peptides consisting of the H3 N-terminal 21 amino acids modified by mono- or di-methylation of Lys[4]. These were the peptides employed in the early studies that led to the discovery of LSD1 function (4, 5). The catalytic efficiency using these two substrates turned out to be very similar (TABLE ONE and Fig. 2*b*), demonstrating that LSD1 does not exhibit a strong preference for mono- *versus* dimethylated H3-K4 substrate. An additional value of the steady-state enzymatic parameters measured with the 21-amino acid peptides was that they provided a benchmark to

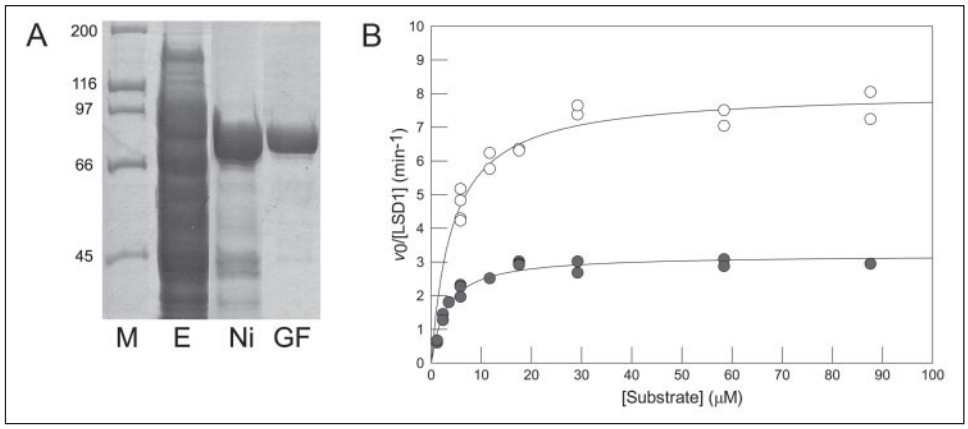

FIGURE 2. **Biochemical characterization of LSD1-Δ184 protein.** *A*, representative protein samples from each stage of the purification analyzed by SDS-PAGE (8% acrylamide). *M*, molecular weight markers; *E*, cell extract; *Ni*, pooled elutions from Ni^{2+}-nitrilotriacetic acid resin; *GF*, pooled elutions from gel filtration. *B*, dependence of initial velocities of reactions of LSD1 on the concentration of 21aa-[monomethyl]K4 (*solid circles*) and 21aa-[dimethyl]K4 (*open circles*) peptides in 50 mM Hepes/NaOH buffer, pH 7.5, at 25 °C (see "Experimental Procedures" and TABLE ONE). The *curves* are the best fit of the data to the Michaelis-Menten equation, $v = V_{max}[S]/(K_m + [S])$. For each substrate concentration, at least two measurements have been done; at low substrate concentrations, the calculated errors were less than 11% for both plots.

TABLE ONE

Substrate specificity of human LSD1

	k_{cat} [a]	K_m [a]	k_{cat}/K_m [a,b]	Percentage[c]
	min^{-1}	μM	$\mu M^{-1}\ min^{-1}$	%
Peptides of 21 amino acids[d]				
21aa-[monomethyl]K4	3.2 ± 0.1	3.0 ± 0.3	1.1 ± 0.12	100
21aa-[dimethyl]K4	8.1 ± 0.2	4.2 ± 0.5	1.9 ± 0.23	173
21aa-[dimethyl]K4 in 80 mM NaCl[e]	7.1 ± 0.6	12.8 ± 2.8	0.55 ± 0.13	50
Peptides of different lengths				
30aa-[monomethyl]K4	2.9 ± 0.1	3.4 ± 0.5	0.8 ± 0.12	73
16aa-[monomethyl]K4	ND[f]	ND	ND	ND
12aa-[monomethyl]K4	ND	ND	ND	ND
8aa-[monomethyl]K4	ND	ND	ND	ND
Epigenetic marks				
21aa-[monomethyl]K4-[monomethyl]K9	5.6 ± 0.2	3.9 ± 0.5	1.4 ± 0.19	130
21aa-[monomethyl]K4-[phospho]S10	ND	ND	ND	ND
21aa-[monomethyl]K4-[acetyl]K9	4.1 ± 0.3	17.5 ± 4.0	0.23 ± 0.06	21

[a] Apparent steady-state kinetic parameters were determined in air-saturated buffer (50 mM Hepes/NaOH, pH 7.5, at 25 °C) by a horseradish peroxidase-coupled assay (5).
[b] Propagation of statistical error was carried out as described (22).
[c] The percentage of activity with reference to that measured with 21aa-[monomethyl]K4.
[d] As a control, the k_{cat} and K_m values for 21aa-[dimethyl]K4 and 21aa-[monomethyl]K4 peptides were measured also by means of a ferricenium assay (5), with ferricenium being a nonphysiological monoelectronic acceptor that reacts directly with the reduced LSD1-bound FAD cofactor. The resulting values were essentially identical to those measured with the peroxidase-coupled assay (not shown).
[e] The assay was performed in 80 mM NaCl, 50 mM Hepes/NaOH, pH 7.5, at 25 °C.
[f] ND, not determined. The activity is absent or barely detectable.

evaluate the activity of LSD1 toward peptides of different lengths or bearing additional covalent modifications (TABLE ONE).

The peptide substrate is rich in charged and titratable groups (the N-terminal amino group; three Arg and four Lys side chains) that in many cases are sites of epigenetic marks (Fig. 1*b*). We have therefore evaluated the effect of pH and ionic strength on the enzymatic activity. Both apparent k_{cat} and k_{cat}/K_m exhibit a bell-shaped pH dependence approaching 0 at low and high pH values (Fig. 3). The k_{cat}/K_m value increases as a group with pK_a of about 7.2 dissociates and decreases as a group with pK_a of about 8.9 becomes deprotonated. k_{cat} shows a similar profile, with pK_a values of 7.4 and 9.2, respectively. The similar pK_a values observed in the k_{cat} and k_{cat}/K_m profiles suggest that they reflect the dissociation state of the same groups and that such groups belong to the enzyme rather than to the peptide substrate (15). However, the possibility cannot be ruled out that the pK_a value of 8.9 observed in the k_{cat}/K_m profile actually reflects the dissociation of one of the Arg or Lys residues of the substrate. Despite the fact that the k_{cat}/K_m and k_{cat} profiles exhibit maximal values at pH 8.0 and 8.4, respectively, the data reported in this paper refer to assays performed at pH 7.5, which is of physiological significance and close to the optimal pH for activity.

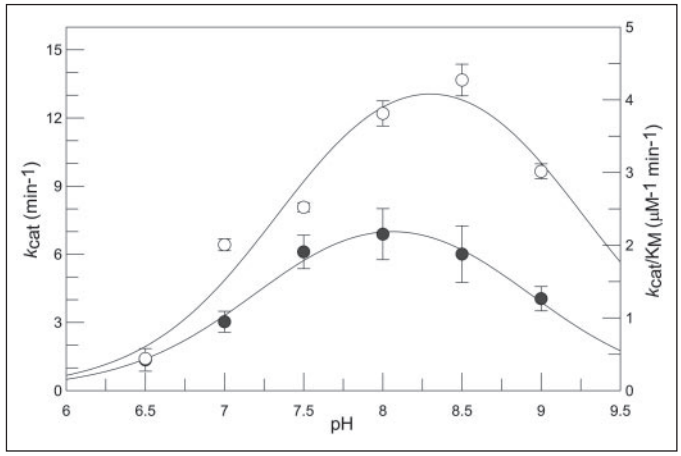

FIGURE 3. **pH dependence of the steady-state kinetic parameters of the 21aa-[dimethyl]K4 demethylation reaction catalyzed by LSD1.** *Filled circles*, k_{cat}/K_m values; *open circles*, k_{cat}. The *curves* show the best fit of the data to a double pK_a model (for k_{cat}/K_m, $pK_{a1} = 7.2 \pm 0.1$, and $pK_{a2} = 8.9 \pm 0.1$; for k_{cat}, $pK_{a1} = 7.4 \pm 0.2$, and $pK_{a2} = 9.2 \pm 0.3$). Propagation of statistical error value was carried out as described (22).

TABLE TWO

Inhibition of human LSD1 by histone H3 peptides

Peptide	K_i[a]
	μM
21aa (product)	1.8 ± 0.6
12aa	199 ± 22
Nter-Δ5–21aa	87 ± 29
21aa-[monomethyl]K4-[phospho]S10	31 ± 5

[a] Enzymatic activities were evaluated in air-saturated buffer (50 mM Hepes, pH 7.5, at 25 °C) by a horseradish peroxidase-coupled assay using a 21-amino acid peptide monomethylated on Lys[4] (5). In all cases, the inhibition was of the competitive type.

A crucial role of ionizable groups is suggested also by the analysis of the effect of the ionic strength on LSD1 catalytic function. The enzyme activity remains essentially constant at NaCl or KCl concentrations up to 50 mM, but it decreases sharply at higher concentrations so that only 10% of residual activity is measured at concentrations greater than 150 mM. The observed decrease of activity is due to a K_m increase, as shown by the fact that at 80 mM NaCl, the K_m value is 3-fold higher than that found in the absence of added salt, whereas the measured k_{cat} values at 0 and 80 mM NaCl are essentially identical (see TABLE ONE). In this respect, the 4-fold lower specific activity previously reported for LSD1 (5) can be ascribed to the ionic strengths of the initial assays, which were carried out in 50 mM sodium phosphate buffer, pH 7.5 (ionic strength = 132 mM) as opposed to 50 mM Hepes/NaOH, pH 7.5 (ionic strength = 22 mM).

Substrate Recognition and Specificity—To define the actual peptide length recognized by LSD1, we studied the ability of the enzyme to demethylate peptides consisting of the N-terminal 8, 12, 16, and 30 amino acids (Fig. 1*b*) and modified by monomethylation of Lys[4] (TABLE ONE). Both k_{cat} and k_{cat}/K_m values for the 30-amino acid substrate were very similar to those measured with the 21-amino acid peptide. Conversely, with the 16-amino acid substrate, the activity became barely detectable, and with shorter peptides no activity could be detected at all. The lack of activity with these "short" peptides was observed also at a pH value of 8.4, which is optimal for catalysis (Fig. 3). The outcome of these experiments indicated that substrate recognition by LSD1 is not confined to the residues neighboring Lys[4], but it requires a rather long peptide emphasizing the exquisite substrate specificity of the protein. LSD1 is normally associated to the CoREST protein, which has been shown to enhance LSD1 activity promoting demethylation of the intact nucleosomal particle (11, 16). CoREST might exert this effect through stabilization of the interaction with the nucleosomal substrate and/or increase of the accessibility of the N-terminal tail.

Enzyme Inhibition—The finding that LSD1 recognizes a long H3 peptide segment suggested the possibility that the reaction product (*i.e.* the demethylated peptide) could bind to the protein. Experimental verification of this hypothesis revealed that the unmodified 21-amino acid peptide is indeed a competitive inhibitor with a K_i of 1.8 μM (TABLE TWO). Consistent with the substrate specificity studies, a shorter 12-amino acid peptide exhibited a >100-fold reduction in affinity (TABLE TWO). Likewise, deletion of the four N-terminal residues from the 21-amino acid peptide resulted in a 40-fold reduction of the inhibitory power (TABLE TWO). These data imply that methylation of Lys[4] is not essential for binding, whereas the affinity is critically dependent on the presence of a sufficiently long segment of the H3 N-terminal tail.

Recently, Metzger *et al.* (13) have reported on an androgen receptor-dependent activity of LSD1. The interaction with the receptor appears to switch the substrate specificity, making LSD1 able to demethylate Lys[9] of H3 (isolated LSD1 does not act on Lys[9]) (4, 5). This activity was shown to be inhibited by pargyline, deprenyl, and clorgyline, which are specific inhibitors of human monoamine oxidase A and/or B (17). We have tested whether these inhibitors act on LSD1, interfering with its H3-K4 demethylase function. No inhibition was detected, and, in addition, none of these compounds induced the characteristic changes in the absorption spectrum of the protein-bound flavin that are found in inhibited monoamine oxidases (data not shown).

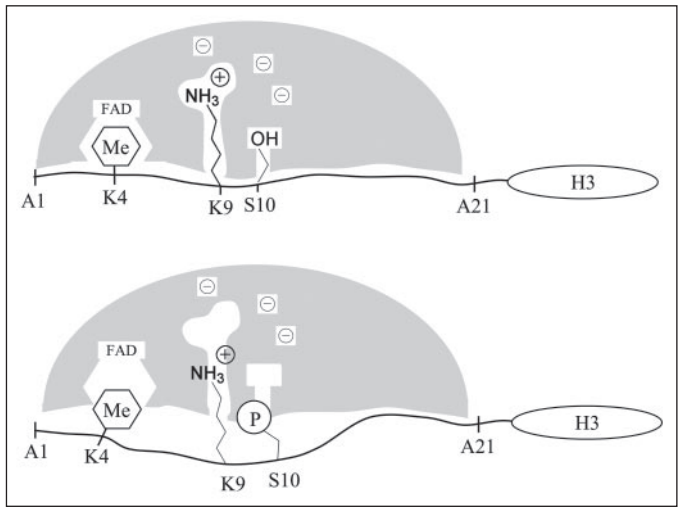

FIGURE 4. **The effect exerted by phosphorylation of Ser[10].** This epigenetic mark does not abolish binding, but it makes the peptide unable to bind in a productive mode. The Lys[9]-Ser[10] pair is predicted to interact with a cluster of hydrogen bond acceptors and/or negatively charged groups on LSD1.

Readout of Multiple Epigenetic Marks—The N-terminal tail of H3 is subject to various covalent modifications, and a crucial question in LSD1 biology is how these epigenetic marks affect the demethylase activity. We have probed LSD1 for its ability to demethylate Lys[4] in the presence of a second modification on the same 21-amino acid peptide substrate. As shown in TABLE ONE, we tested the effects of methylation and acetylation of Lys[9] and phosphorylation of Ser[10]. Methylation of Lys[9] did not affect activity, whereas Lys[9] acetylation caused an almost 6-fold increase in the K_m value, indicating that this covalent modification significantly affects the substrate binding affinity. Even more dramatic is the effect of Ser[10] phosphorylation, which totally suppressed the ability of the peptide to function as substrate. Taken together, these data are consistent with the notion that electrostatic interactions are especially relevant in the enzyme-substrate recognition. Methylation of Lys[9], which does not neutralize the Lys positive charge, has no effect on enzymatic reaction. Conversely, neutralization of Lys[9] positive charge by acetylation reduces affinity, whereas introduction of the negatively charged phosphate group on Ser[10] completely abolishes activity. These findings predict that the Lys[9]-Ser[10] pair binds to a cluster of hydrogen bond acceptors and/or negatively charged groups that enable LSD1 to sense the presence of various covalent modifications on this locus (Fig. 4). A fundamental conclusion gathered from these experiments is that LSD1 is actually capable of reading the histone code in that it discriminates between peptides bearing different covalent modifications on their side chains to the point that a single mark makes the difference between a reactive and a nonreactive peptide.

Interestingly, we found that the phosphorylated Ser[10] peptide is a moderately effective inhibitor of the enzyme (K_i of 31 μM) (TABLE TWO). This observation suggests that the phosphorylated peptide is still able to bind (although weakly) to LSD1, but its binding mode is nonproductive so that it cannot be oxidized. In other words, the phos-

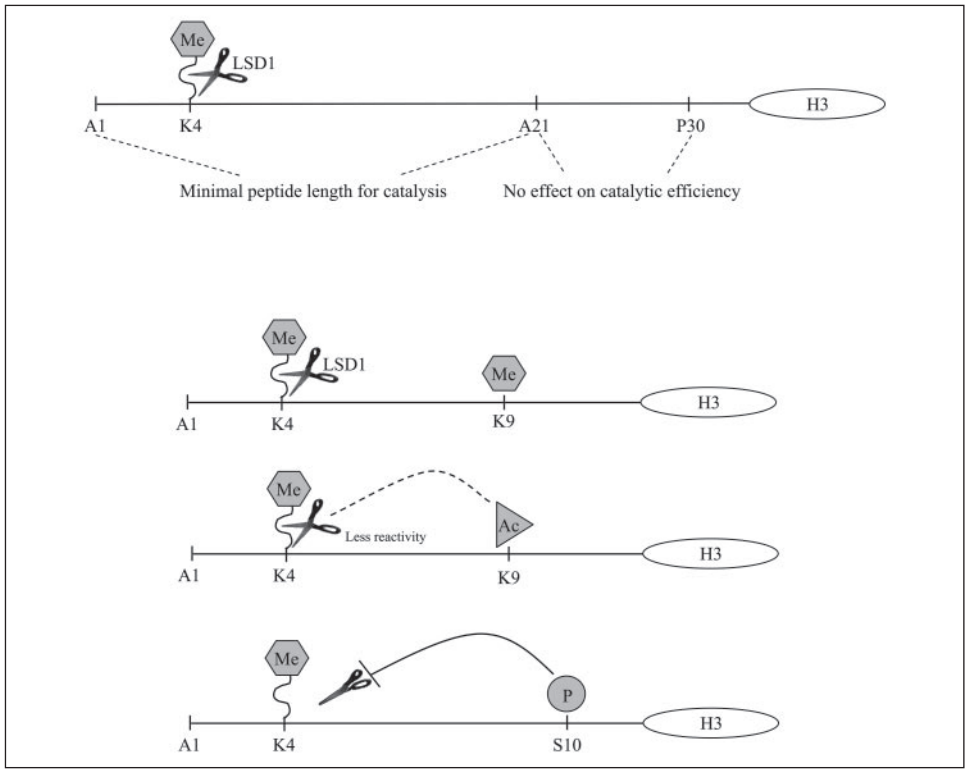

FIGURE 5. **Reading of the histone code by human LSD1: A minimal peptide length of 21 amino acids is required for activity.** LSD1 does not act on a peptide that is phosphorylated on Ser^{10} (*S10*), whereas acetylation of Lys^9 (*K9*) reduces enzymatic activity. Conversely, methylation of Lys^9 does not appear to affect catalysis and binding.

phate group on Ser^{10} appears to prevent the catalytically competent positioning of the methylated Lys^4 inside the active site (Fig. 4).

DISCUSSION

The main theme emerging from these data is that LSD1 is finely tuned and highly specific. This finding has profound implications for the control of histone H3 demethylation and its role in chromatin biology. A first important observation is that LSD1 does not have a strong preference for mono- or dimethylated H3-K4. This feature contrasts with the properties of many lysine methyltransferases that specifically perform the addition only of the first, second, or third methyl group on a Lys residue (18). The fact that LSD1 acts with similar efficiency (TABLE ONE) on mono- and dimethylated substrates indicates that the *in vivo* function of LSD1 is to reset H3-K4 to its demethylated "ground" state.

We have shown that an H3 peptide must contain the first ~20 N-terminal amino acids of H3 in order to be a substrate. The sequence of this polypeptide stretch has many charged residues, and electrostatic interactions are likely to dominate the protein-substrate association, providing a rationale for the high sensitivity of K_m to ionic strength. This ability of recognizing a "long" and "heavily charged" segment of the histone N-terminal tail enables LSD1 to detect the presence of multiple post-translational modifications written in the H3 tail (Fig. 5). In this regard, several important functional properties have been unraveled. (i) LSD1 has an exquisite specificity. Although the site of oxidative attack is Lys^4, the enzyme senses covalent modifications on neighboring residues. (ii) The charge distribution on the histone N-terminal tail appears to be a key determinant in the readout of the histone code by LSD1, as exemplified by the differential effects of methylation and acetylation of Lys^9 on enzyme activity. (iii) The Lys^4 demethylase activity of LSD1 is not affected by methylation of Lys^9, which is generally known to cause gene repression. (iv) Acetylated Lys^9 and phosphorylated Ser^{10} cross-talk and act synergistically in the Lys^4 demethylation process. Ser^{10} dephosphorylation is a prerequisite for LSD1 function, whereas Lys^9 deacetylation significantly improves activity mainly through an increase in substrate affinity. (v) These features provide insight into the order and interplay of the events that lead to transcriptional repression. Dephosphorylation of Ser^{10} by a phosphatase must precede H3 demethylation catalyzed by LSD1, emphasizing the role of Ser^{10} phosphorylation/dephosphorylation in triggering the cascades of events that lead to transcriptional activation/repression. Lys^9 deacetylation by HDAC1/2 (which are typically associated with LSD1) makes H3 more susceptible to LSD1-catalyzed demethylation.

The activity of LSD1 can be modulated by its associated protein factors (11, 13, 16), and the enzyme specificity observed on free peptides might be different when chromatin and nucleosomes are used as substrates. However, it is of notice that the lower efficiency observed with a peptide acetylated on Lys^9 is consistent with the finding by Shi *et al.* (11) that LSD1 is more active toward hypoacetylated nucleosomes.

LSD1 binds with significant affinity (TABLE TWO) to a demethylated H3 peptide, as indicated by an inhibition constant that compares with the K_m for the peptide substrates. The measured affinity is similar to the affinity measured for other H3-binding proteins such as the chromodomain-containing proteins HP1 (19) and Polycomb (20). This finding raises the intriguing hypothesis that LSD1 plays a double role, namely that of Lys^4-specific demethylase and of H3 N-terminal tail binder. LSD1 is member of the co-repressor complex CoREST-HDAC1/2, a module that has been found in association with several large multiprotein complexes. The finding that LSD1 has significant affinity for the H3 peptide indicates that it could serve as a docking module for the stabilization of the associated corepressor complex(es) on chromatin. At least in one case, it has been demonstrated that the CoREST corepressor complex remains bound to chromatin after the release of the DNA-binding protein responsible for its initial recruitment (21).

The main conclusion from these studies is that LSD1 is not only a chromatin-modifying enzyme, but it is able to interpret histone marks on the Lys^9-Ser^{10} locus and to stabilize the co-repressor complex by binding to the demethylated H3. Knowledge of LSD1 substrate specific-

ity properties will provide a framework for future inhibitor design studies.

Acknowledgments—The continuous interest of A. Coda, E. Ginelli, and G. Mandel is gratefully acknowledged. We thank N. Landsberger for reading the manuscript.

REFERENCES

1. Biel, M., Wascholowski, V., and Giannis, A. (2005) *Angew. Chem. Int. Ed. Engl.* **44,** 3186–3216
2. Fischle, W., Wang, Y., and Allis, C. D. (2003) *Nature* **425,** 475–479
3. Jenuwein, T., and Allis, C. D. (2001) *Science* **293,** 1074–1080
4. Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., Casero, R. A., and Shi, Y. (2004) *Cell* **119,** 941–953
5. Forneris, F., Binda, C., Vanoni, M. A., Mattevi, A., and Battaglioli, E. (2005) *FEBS Lett.* **579,** 2203–2207
6. Sims, R. J. 3rd, Nishioka, K., and Reinberg, D. (2003) *Trends Genet.* **19,** 629–639
7. Bannister, A. J., and Kouzarides, T. (2005) *Nature* **436,** 1103–1106
8. Humphrey, G. W., Wang, Y., Russanova, V. R., Hirai, T., Qin, J., Nakatani, Y., and Howard, B. H. (2001) *J. Biol. Chem.* **276,** 6817–6824
9. Ballas, N., Battaglioli, E., Atouf, F., Andres, M. E., Chenoweth, J., Anderson, M. E., Burger, C., Moniwa, M., Davie, J. R., Bowers, W. J., Federoff, H. J., Rose, D. W., Rosenfeld, M. G., Brehm, P., and Mandel, G. (2001) *Neuron* **31,** 353–365
10. Shi, Y., Sawada, J., Sui, G., Affar, B., Whetstine, J. R., Lan, F., Ogawa, H., Luke, M. P., Nakatani, Y., and Shi, Y. (2003) *Nature* **422,** 735–738
11. Shi, Y. J., Matson, C., Lan, F., Iwase, S., Baba, T., Shi, Y. (2005) *Mol. Cell.* **19,** 857–864
12. Binda, C., Mattevi, A., and Edmondson, D. E. (2002) *J. Biol. Chem.* **277,** 23973–23976
13. Metzger, E., Wissmann, M., Yin, N., Muller, J. M., Schneider, R., Peters, A. H., Gunther, T., Buettner, R., and Schule, R. (2005) *Nature* **437,** 436–439
14. Aliverti, A., Curti, B., and Vanoni, M. A. (1999) *Methods Mol. Biol.* **131,** 9–23
15. Segel, I. H. (1975) in *Enzyme Kinetics*, John Wiley & Sons, Inc., New York
16. Lee, M. G., Wynder, C., Cooch, N., and Shiekhattar, R. (2005) *Nature* **437,** 432–435
17. Edmondson, D. E., Mattevi, A., Binda, C., Li, M., and Hubalek, F. (2004) *Curr. Med. Chem.* **11,** 1983–1993
18. Cheng, X., Collins, R. E., and Zhang, X. (2005) *Annu. Rev. Biophys. Biomol. Struct.* **34,** 267–294
19. Jacobs, S. A., Taverna, S. D., Zhang, Y., Briggs, S. D., Li, J., Eissenberg, J. C., Allis, C. D., and Khorasanizadeh, S. (2001) *EMBO J.* **20,** 5232–5241
20. Fischle, W., Wang, Y., Jacobs, S. A., Kim, Y., Allis, C. D., and Khorasanizadeh, S. (2003) *Genes Dev.* **17,** 1870–1881
21. Ballas, N., Grunseich, C., Lu, D. D., Speh, J. C., and Mandel, G. (2005) *Cell* **121,** 645–657
22. Bevington, P. H. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, pp. 56–62, McGraw-Hill, New York