Histone demethylation catalysed by LSD1 is a flavin-dependent oxidative process

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Abstract Lysine-specific histone demethylase 1 (LSD1) is a very recently discovered enzyme which specifically removes methyl groups from Lys4 of histone 3. We have addressed the functional properties of the protein demonstrating that histone demethylation involves the flavin-catalysed oxidation of the methylated lysine. The nature of the substrate that acts as the electron acceptor required to complete the catalytic cycle was investigated. LSD1 converts oxygen to hydrogen peroxide although this reactivity is not as pronounced as that of other flavin-dependent oxidases. Our findings raise the possibility that in vivo LSD1 might not necessarily function as an oxidase, but it might use alternative electron acceptors.

Keywords: Flavoenzyme; Amine oxidase; KIAA0601; Histone methylation; Chromatin remodelling

1. Introduction

Histones are substrates for a number of covalent modifications that control chromatin state and gene expression. Methylation of lysine residues on the N-terminal tails of histones is one of the elements that define the so-called ''histone code'' [1]. Very recently, Shi et al. [2] have described the discovery of lysine-specific histone demethylase 1 (LSD1), an enzyme which has remained elusive for decades and whose existence has even been questioned. This protein was shown to act on dimethylated Lys4 of histone 3 (H3-K4). The LSD1-catalysed reaction regenerates the methyl-free lysine together with release of formaldehyde (Fig. 1, [2]). LSD1 comprises an N-terminal SWIRM domain and a C-terminal flavin domain which shows homology to members of the amine oxidase family (Fig. 2). These enzymes catalyse the oxidative deamination of compounds that contain primary, secondary or tertiary amines [3]. LSD1 (also known as KIAA0601) has been typically found in association with CoREST and histone deacetylases 1 and 2 [4,5]. These proteins form a highly conserved module [6–8] that has been shown to be part of several megadalton co-repressor complexes, which are proposed to operate in the context of a stable and extended form of repression through silencing of entire chromatin domains [9,10].

Here, we report on the production, purification of two LSD1 truncated forms, lacking the first 157 (Δ157) and 184 (Δ184) amino acids (Fig. 2), respectively, and we demonstrate that: (i) the C-terminal 702 residues of the protein contain the fully functional histone demethylation site; (ii) histone demethylation involves the enzyme-bound FAD cofactor; (iii) the protein exhibits oxidase activity but it can function also with a synthetic mono-electronic acceptor, raising the hypothesis that in vivo enzyme reoxidation might not be an oxygen-mediated process and it can involve alternative electron acceptors.

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Abbreviations: LSD, lysine-specific histone demethylase; Δ184, human LSD1 mutant harbouring a deletion of the N-terminal 184 amino acids; Δ157, mutant harbouring a deletion of the N-terminal 157 amino acids; H3-K4, Lys4 of histone 3; H3-K9, Lys9 of histone 3

2. Methods

2.1. Protein expression and purification

All chemicals were purchased from Sigma–Aldrich unless specified. Analysis of the amino acid sequence (Fig. 2) with bioinformatics tools [11] predicts that the N-terminal 150 amino acids lack secondary structures. We produced two mutant proteins that lack the first 157 (Δ157) and 184 (Δ184) amino acids. The cDNA obtained by PCR coding for mutant harbouring a deletion of the N-terminal 157 amino acids (Δ157) and human LSD1 mutant harbouring a deletion of the N-terminal 184 amino acids (Δ184) mutants were sub-cloned into a pET28b vector and expressed in *Escherichia coli* cells (BL21-DE3). Cells were grown in a 5 l Bioflo 3000 fermentor at 37 °C up to OD_{600} of 0.9, temperature was lowered to 25 °C, and cells were harvested 6 h after the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside. Cells (15 g) were resuspended in 75 ml lysis buffer (50 mM sodium phosphate, pH 7.8, 5% glycerol, 200 mM NaCl, 1 mM phenyl-methylsulphonil fluoride). The suspension was passed through a French press and cell debris was removed by centrifuging at 70 000 × *g* at 4 °C. The supernatant was incubated for 3 h at 4 °C with 3 ml of a 50% slurry Ni-NTA (Qiagen) equilibrated in lysis buffer. The resin was poured onto a column, washed with 50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, 5% glycerol and the protein was eluted with 50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, 5% glycerol. The fractions enriched in LSD1 were pooled and chromatographed on a MonoS HR 5/5 column (Amersham) equilibrated in 50 mM sodium malonate, pH 5.2, 200 mM NaCl, 10% glycerol. Elution was performed with a 15 ml linear gradient of NaCl (200 mM–1 M). LSD1-containing fractions were concentrated by Centricon30 centrifugal concentrators, gel filtered on a Superdex200 HR 10/30 column equilibrated and eluted with 25 mM potassium phosphate, pH 7.2, 5%

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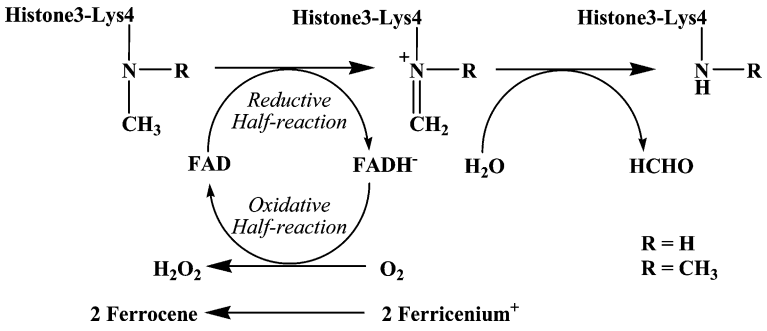

Fig. 1. Scheme of the oxidative demethylation reaction catalysed by the flavin-dependent amine oxidase LSD1.

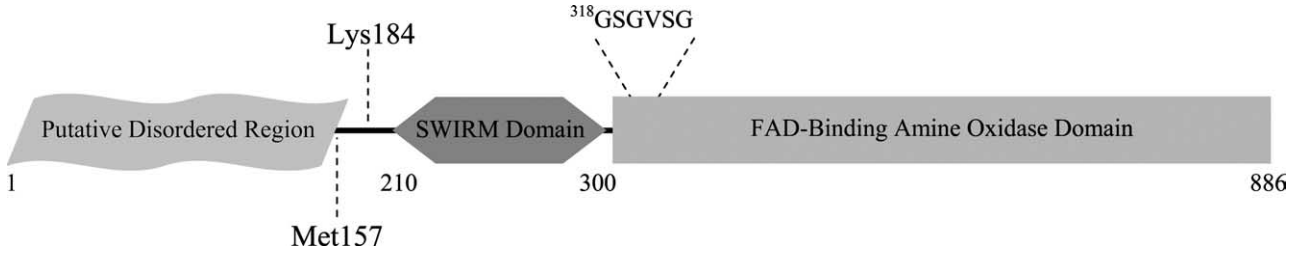

Fig. 2. Domain organisation of human LSD1. We use as reference, the sequence of human LSD1 deposited in the NCBI database under the accession code BAA25527 (886 residues). Residue Met35 of this sequence corresponds to the first amino acid of the protein characterised by Shi et al. [2]. Met157 and Lys184 are the sites of N-terminal truncation in the Δ157 and Δ184 mutants. The GXGXXG sequence represents the highly conserved motif located at the N-terminus of the flavin-binding domain.

glycerol. Both LSD1 proteins recovered from this column were estimated to be greater than 95% homogeneous as judged by SDS–PAGE analysis. The A_{280}/A_{458} was about 10 for both mutants. Purified enzymes were stored on ice.

2.2. Cofactor analysis

Δ184 and Δ157 (10 μM) aliquots were incubated at 100 °C in the dark for 15 min and denatured protein was removed by centrifugation. The spectrum of the supernatant exhibited the typical features of free flavin with an absorption peak at 450 nm (rather than 458 nm of the LSD1-bound flavin). This indicates the non-covalent nature of cofactor binding. The method of Aliverti et al. [12] was employed to discriminate between FAD and FMN.

2.3. Enzyme reactivity

Δ184 (10 μM in 25 mM potassium phosphate, pH 7.2, 5% glycerol) was anaerobically incubated with 20 μM dimethylated H3-K4 peptide (from Upstate Group Inc.) and spectral changes were monitored by using a HP 8453 UV/Vis diode-array spectrophotometer. Enzyme reoxidation was performed by either anaerobic addition of ferricenium hexafluorophosphate [13,14] or exposure to air. Ferricenium stock solutions were standardised spectrophotometrically at 617 nm by using an extinction coefficient $\varepsilon_{617} = 410\ M^{-1}\ cm^{-1}$. Oxygen and ferricenium reactivity was analysed also with reference to enzyme that has been photoreduced in the presence of 1 μM deazaflavin and 5 mM EDTA [15].

2.4. Activity assays

Time-course measurements were performed under aerobic conditions by using a Cary 100 UV/Vis spectrophotometer. The reaction was started by adding the protein at two different concentrations (0.5 and 3.1 μM in 25 mM potassium phosphate, pH 7.2, 5% glycerol in a final volume of 100 μl). In the peroxidase-coupled assay [16], the mixture contained 0.1 mM 4-aminoantipyrine, 1 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 2.8 μM horseradish peroxidase, and 17 μM dimethylated H3-K4 peptide; absorbance changes were monitored at 515 nm ($\varepsilon_{515} = 26\,000\ M^{-1}\ cm^{-1}$). In the ferricenium assay, the mixture contained 70 μM ferricenium hexafluorophosphate and 17 μM dimethylated H3-K4 peptide. Decrease of absorbance at 300 nm was monitored ($\varepsilon_{300} = 4300\ M^{-1}\ cm^{-1}$ [14]).

3. Results and discussion

We refer to the experiments that have been performed using the Δ184 mutant; the Δ157 protein exhibits virtually identical reactivity and spectroscopic properties (not shown).

3.1. LSD1 reactivity with H3-K4 dimethylated peptide

Purified LSD1 mutants were found to contain a bound flavin cofactor (Fig. 3A), which was shown to be FAD. Protein denaturation led to cofactor release, implying that it is not covalently bound. LSD1 was anaerobically incubated with dimethylated H3-K4 peptide (Fig. 3A) leading to the reduction of the enzyme-bound cofactor as indicated by the bleaching of the absorption peak at 458 nm. Reaction with the substrate resulted in the accumulation of the two-electron fully reduced flavin without any detectable formation of one-electron reduced forms of the cofactor. These features demonstrate that the first step in the demethylation reaction is the flavin-mediated two-electron oxidation of the methylated lysine; this leads to formation of the corresponding imine molecule coupled to cofactor reduction (Fig. 1). To complete substrate demethylation, hydrolysis of the imine must occur. It remains to be established whether this hydrolytic step is catalysed by the enzyme with release of the demethylated peptide, or hydrolysis is a non-enzymatic process that takes place in solution after dissociation of the enzyme–product complex.

3.2. LSD1 has oxidase activity

Completion of the catalytic cycle requires reoxidation of the flavin cofactor (oxidative half-reaction; Fig. 1). In this regard, the key question concerns the nature of the physiological electron acceptor. We have probed the enzyme for its ability to react with molecular oxygen. Exposure of the reduced enzyme to

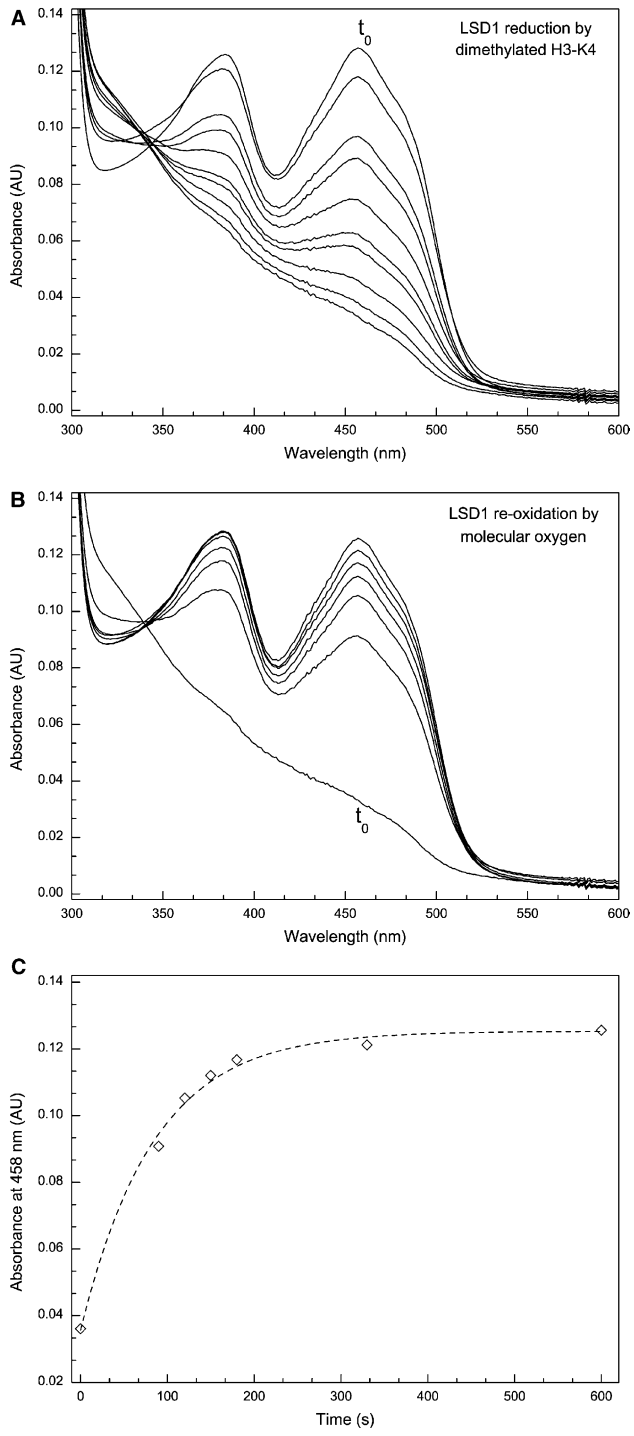

Fig. 3. Reactivity of LSD1. (A) Reduction by 20 μM H3-K4 dimethylated peptide under anaerobic conditions. The experiment was carried out using Δ184 mutant (10 μM). The spectral changes in the UV/Vis range indicate the occurrence of the flavin reduction. From the bleaching of the 458 nm peak it can be estimated that >80% of the enzyme is reduced. Spectra were measured at 0 (oxidised enzyme), 2, 2.5, 3, 7, 10, 13, 27, 55, and 152 min after anaerobic incubation with the peptide. (B) The anaerobically reduced enzyme (see A) was exposed to air. The oxygen reacts with the flavin leading to enzyme re-oxidation. Spectra were measured at 0 (reduced enzyme), 1.5, 2, 2.5, 3, 5.5, and 10 min after exposure to air. (C) Time-course of the re-oxidation process. The curve shows the fit of the data to a single exponential process according to the following equation: $\text{Abs} = 0.125 - 0.90 \cdot e^{-0.0.12 \cdot t}$ with t expressed as seconds.

air led to reoxidation of the cofactor (Fig. 3B), resulting in an absorption spectrum that was indistinguishable from that of the native enzyme. Completion of the reoxidation process took several minutes (Fig. 3C). Oxygen reactivity was also probed using enzyme that was reduced by light exposure in the presence of deazaflavin and EDTA [15]. The time-course of the oxidation reaction was essentially identical to that observed with the substrate-reduced enzyme, indicating that oxygen reactivity is not greatly affected by the presence of the reaction substrate or product. Demonstration that oxygen can function as electron acceptor prompted us to check whether oxygen-mediated flavin reoxidation produces H_2O_2. For this purpose, the enzyme was incubated with dimethylated H3-K4 peptide and the production of H_2O_2 was monitored with a peroxidase-coupled assay (Fig. 4) [16]. Taken together, these experiments demonstrate that histone demethylation by LSD1 is an oxidative process that can use oxygen as electron acceptor with production of H_2O_2.

3.3. Reactivity with a synthetic electron acceptor

We investigated the reactivity of LSD1 with alternative electron acceptors to be employed as substrates in the oxidative half-reaction (Fig. 1). Ferricenium has been often used a synthetic electron acceptor to assay for the activity of flavin-dependent oxidoreductases [14]. Ferricenium is reduced to ferrocene (Fig. 1) in a one-electron reaction that can be conveniently monitored by UV/Vis absorption spectroscopy. Anaerobic addition of ferricenium to reduced LSD1 led to reoxidation of the protein (Fig. 5A). In particular, an equimolar concentration of ferricenium led only to partial reoxidation, consistently with the fact that it is a one-electron acceptor. Incubation with more than a twofold molar excess was necessary to completely regenerate the oxidised enzyme (Fig. 5A). An important feature in the reaction is that the ferricenium-mediated oxidation of LSD1 is essentially instantaneous, clearly differing from the slower

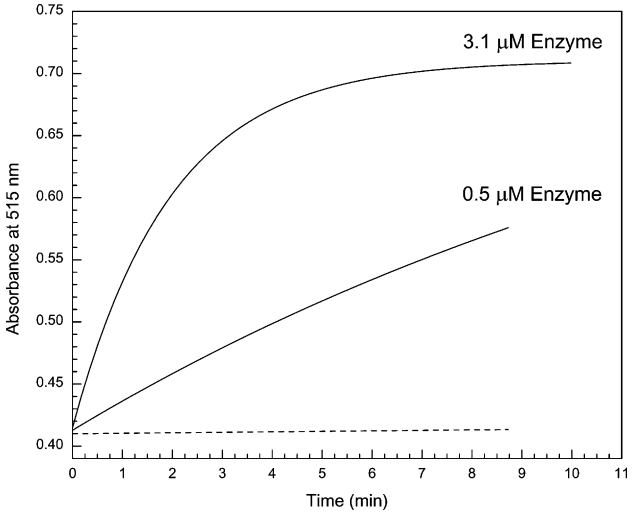

Fig. 4. Demethylase activity was detected using a peroxidase-coupled assay by measuring the increase in absorbance at 515 nm. The assay was carried out using 17 μM H3-K4 dimethylated peptide at two protein concentrations (0.5 and 3.1 μM; solid lines). Under these conditions, the apparent enzyme turnover number is 1.8 min^{-1}. The assay was performed also using 17 μM H3-K9 dimethylated peptide at 3.1 μM protein concentration (dashed line). No activity was detected indicating that H3-K9 is not a substrate of LSD1.

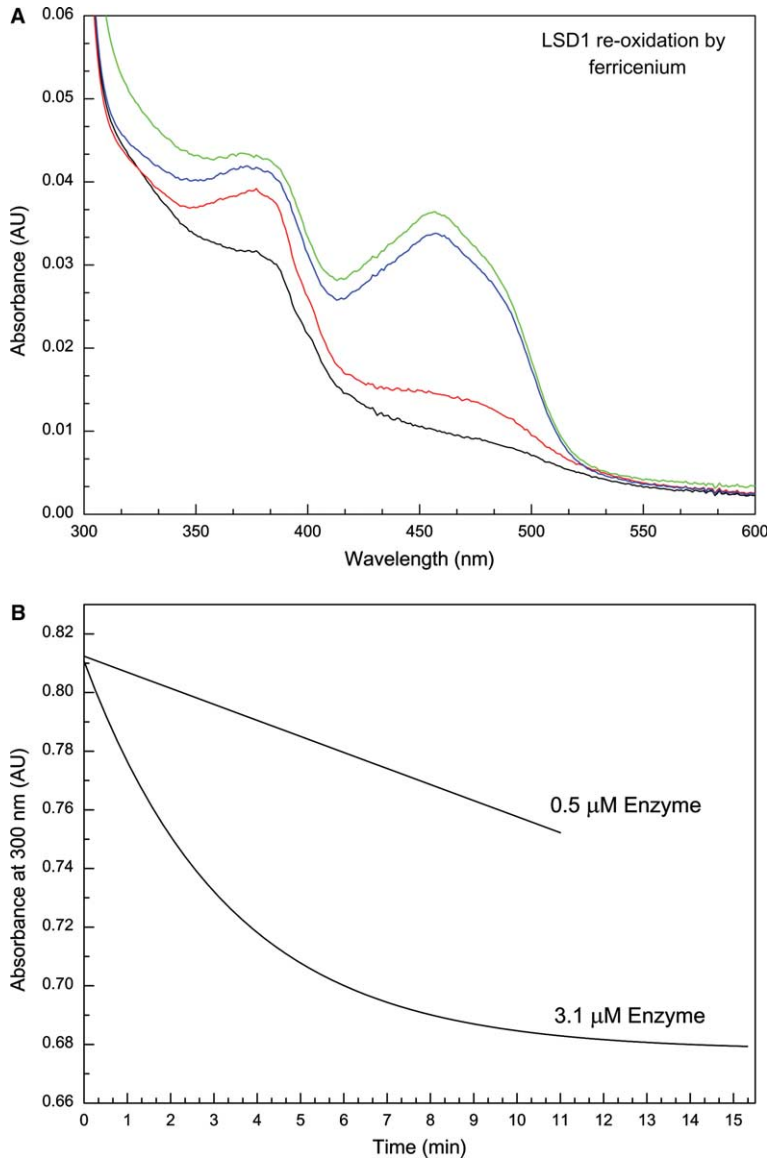

Fig. 5. Reoxidation of Δ184 LSD1 mutant by ferricenium. (A) Spectrum of the anaerobically two-electron reduced enzyme (black; 3.0 μM). After incubation with an equimolar concentration of ferricenium (red) the enzyme is partly reoxidised, in keeping with the fact that ferricenium is a one-electron acceptor. Complete reoxidation is obtained only after addition of a second aliquot of ferricenium (blue) that raises its concentration to 8.5 μM. Exposure to air does not cause any further alteration of the spectrum (green) demonstrating that ferricenium is able to fully reoxidise the enzyme. The reoxidation by ferricenium occurs very rapidly after incubation indicating fast reactivity. (B) Activity measurement using ferricenium as electron acceptor with 17 μM H3-K4 dimethylated peptide at two protein concentrations (0.5 and 3.1 μM). Ferricenium reduction is detected by measuring the decrease of absorbance at 300 nm. In these conditions, the apparent enzyme turnover number is 1.28 min^{-1}.

oxidation process that uses molecular oxygen as substrate (Fig. 3C).

3.4. Demethylase activity assays and reaction specificity

The described experiments suggest two convenient methods to assay the demethylase activity of LSD1 that are based on the ''oxygen reactivity'' and ''ferricenium reactivity'', respectively. The oxidase activity of LSD1 generates H_2O_2, whose formation can be detected with an enzyme-coupled assay [16] (Fig. 4). Ferricenium has a characteristic absorption spectrum that changes upon reduction to ferrocene. This feature combined with the effectiveness of ferricenium as electron acceptor provides another convenient way to assay for demethylase activity (Fig. 5B).

Making use of these two assays, we have studied the specificity of the oxidative demethylation reaction catalysed by LSD1. In agreement with Shi et al. [2], we did not observe any activity on peptides dimethylated at Lys9 rather than Lys4 (Fig. 4). In addition, the enzyme did not exhibit any detectable activity in the presence of methyllysine, trimethylamine, methylglycine, and methylarginine. These findings imply that LSD1 is not responsible for the activity that was measured by Kim et al. [17] who first reported on a demethylase acting on histones as well as on free methyllysine. We also probed the enzyme for the ability to oxidise compounds that are substrates of the homologous enzymes polyamine oxidase (spermine, spermidine, and acetylated derivatives), putrescine oxidase (1,4-diaminobutane), and monoamine oxidase (aromatic amines

including benzylamine, tyramine, and amphetamine). None of these compounds exhibited any reactivity. Likewise, we have tested various covalent and non-covalent inhibitors of monoamine oxidase and polyamine oxidase [3,18] for their ability to inhibit LSD1 activity or to act as LSD1 substrates. Also for these molecules we could not find any functional effect. LSD1 is very specific for H3-K4 substrate unlike other amine oxidases such as polyamine oxidase and monoamine oxidase that exhibit relatively broad specificities. Our findings predict that the substrate/inhibitor-binding site of LSD1 will substantially differ from that of homologous amine oxidases of the flavin class [3].

4. Conclusions

We have shown that removal of the methyl group(s) from H3-K4 is an oxidative process: the substrate is oxidised by FAD to generate the corresponding imine that is subsequently hydrolysed. Thus, LSD1 behaves like a typical flavoenzyme of the oxidoreductase/oxidase class that catalyses the two-electron oxidation of its substrate. The fact that both N-terminally truncated mutants are competent in specific histone demethylation and both tightly bind FAD indicates that the N-terminal amino acids are not crucial for activity and cofactor binding. They might contribute to the recruitment and binding of other protein partners that are part of repression complexes.

An intriguing question about LSD1 function concerns the nature of the physiological substrate which acts as electron acceptor to complete the catalytic cycle. We have shown that LSD1 has oxidase activity. However, generation of hydrogen peroxide in the chromatin environment might favour oxidative damage of DNA and might be harmful. Thus, the possibility exists that at least under certain conditions, molecules other than oxygen can function as electron acceptors. The fact that oxygen reactivity is not especially pronounced (Fig. 3C) when compared to that of other flavin-dependent oxidases supports this hypothesis.

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