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Abstract

DJ-1 is a conserved, ubiquitous protein associated to a large number of intracellular processes. Human DJ-1 has been linked to several pathologies, including hereditary forms of Parkinson's disease, cancer, and amyotrophic lateral sclerosis. Several cytoprotective functions of DJ-1 have been reported, however its actual mechanisms of action remain elusive. *In vitro*, DJ-1 has been shown to bind zinc and copper(II) at its active site, which contains a conserved cysteine (C106), and copper(I) at a different binding site. C106 is essential to DJ-1 function, and is easily oxidized upon oxidative stress. Here, we investigated the metal binding and redox properties of DJ-1 in living human cells by in-cell NMR. Intracellular DJ-1 is surprisingly free from interactions with any other cellular components and as such is clearly detectable by NMR. Metal-bound forms of DJ-1 were not observed upon treating the cells with excess zinc or copper. No copper binding was observed when co-expressing DJ-1 with the copper chaperone for superoxide dismutase 1 (SOD1). Co-expression of DJ-1 with SOD1 itself did not promote copper binding to SOD1, excluding a previously suggested function of DJ-1 as a copper chaperone. Overall, our data do not support the role of DJ-1 as a metalloprotein. Conversely, oxidative treatment to the cells caused the complete and selective oxidation of C106 to sulfinic acid, consistent with the reported role of DJ-1 as a redox sensor.

Keywords

In-cell NMR; DJ-1; deglycase; metalloprotein; cysteine sulfinic acid

Introduction

DJ-1 is a homodimeric protein encoded by the *Park7* gene, ubiquitously expressed and highly conserved in all biological kingdoms. It is present in the cytoplasm, in the nucleus and in mitochondria. Human DJ-1 was first identified as an oncogene [1], as its expression is altered in numerous cancers, including breast, lung, esophageal and prostate cancers [2]. Mutations in the DJ-1 sequence are associated with recessive, early-onset Parkinson's disease [3, 4]. DJ-1 is also implicated in ischemic injury and amyotrophic lateral sclerosis and androgen receptor regulation [5]. Furthermore, DJ-1 binds RNA transcripts potentially involved in many cellular processes, both *in vivo* and *in vitro* [6].

DJ-1 is implicated in many processes, including transcriptional regulation, reaction against oxidative stress, and mitochondrial regulation [7]. Several functions have been attributed to DJ-1, including proteasome regulation [8], chaperone activity towards α-synuclein [9, 10], protease activity towards transthyretin [11–13], and glyoxalase activity [14]. DJ-1 has been shown to act as an intracellular deglycase, reacting with methylglyoxal- and glyoxal-glycated proteins [15]. Very recently, DJ-1 has been also shown to act as DNA deglycase, a long sought-after enzyme catalyzing the repair of methylglyoxal- and glyoxal-glycated guanine [16]. While these recent findings indicate that DJ-1 has a major cytoprotective role, it is not yet clear how it exerts this function [17].

Each DJ-1 monomer is a 20 kDa globular protein containing two exposed cysteine residues: C53 and C106. C106 resides in a so-called nucleophilic elbow, which constitutes the active site of the protein, and is easily oxidized by reactive oxygen species (ROS) due to its low thiol pKa value [18]. Multiple oxidation states of C106 have been reported (mainly $–SO_2H$ and $–SO_3H$) in response to oxidative stress [19–21]. Mutation of C106 results in the loss of most of the putative DJ-1 functions [20, 22, 23]. Consequently, redox-sensing and ROS-quenching activities of DJ-1, by direct reaction with C106, have been proposed [7, 22, 24].

DJ-1 has also been shown to bind copper, zinc and mercury *in vitro* [25]. Binding of these toxic metals would confer DJ-1 a protective function against metal-induced cytotoxicity. Zinc binding occurs with low micromolar affinity at the protein active site, with E18, C106 and two water molecules as ligands. Zinc is bound with higher affinity with respect to other divalent metal ions [26]. For copper, two distinct binding

modes have been reported: Cu(II) binds to the same active site as zinc [27], whereas one Cu(I) per monomer binds with higher affinity at the homodimer interface, with two C53 residues, one from each monomer, as ligands [28]. It has been suggested that DJ-1 might act as copper chaperone for superoxide dismutase 1 (SOD1), in place of the known metallochaperone for SOD1, CCS [27, 29].

While potentially relevant, most of the available data on DJ-1 metal binding were obtained *in vitro*, in the absence of the physiological competitors present in the cell. In parallel, while the structure of DJ-1 upon oxidation of C106 has been extensively characterized *in vitro*, the actual oxidation state of C106 in cells exposed to oxidizing agents has not been clearly defined. In this work, we sought to clarify some of these aspects by in-cell NMR [30, 31]. We applied an established protocol to observe protein structural changes at the residue level in the cytoplasm of live human cells [32, 33]. By comparison with *in vitro* NMR data, we could analyze the change in conformation of intracellular DJ-1 based on chemical shift perturbation, that is extremely sensitive to metal binding and changes in redox state, as a result of different cell culture conditions (i.e. addition of metal ions, treatment with H_2O_2). In human cells, we did not observe any metal-bound form of DJ-1, neither with zinc nor with copper in any oxidation state. Consistently, we did not observe copper metallation of SOD1 upon co-expression of DJ-1 in copper-treated cells. These data pointed us to reconsider the potential role of DJ-1 as a metallochaperone. Conversely, DJ-1 was efficiently oxidized upon treatment with H_2O_2 and a single species was produced, corresponding to the C106–sulfinic acid state, thereby suggesting a physiological function for DJ-1 oxidation.

Materials and methods

Gene cloning

The plasmid “DJ-1 TEV site pET15b”, used for bacterial expression, was a gift from Mark Wilson (Addgene plasmid # 60687). For mammalian expression, the WT DJ-1 gene was amplified by PCR and cloned into the pHLsec vector [34] between EcoRI and XhoI restriction enzyme sites. The clone was verified by DNA sequencing. pHLsec vectors encoding WT human SOD1 and CCS had been previously obtained [32].

Human cell culture and transfection

HEK293T (ATCC CRL-3216) cells were maintained in DMEM high glucose (Life Technologies) supplemented with L-glutamine, antibiotics (penicillin and streptomycin) and 10% FBS (Gibco) in uncoated 75 cm^2 plastic flasks and incubated at 37 °C, 5% CO2 in a humidified atmosphere. The cells were transiently transfected with the pHLsec plasmid containing the gene of interest using polyethylenimine (PEI), as previously described [32, 33]. For co-expression of two proteins, cells were transfected with a mixture of pHLsec plasmids containing the genes of interest. [U-^{15}N]-BioExpress6000 medium (Cambridge Isotope Laboratories) was used to produce [U-^{15}N]-labeled samples, supplemented with 2% FBS and antibiotics. For [^{15}N]cysteine labelling, a reconstituted medium was prepared following the reported composition of DMEM (Sigma), in which [^{15}N]cysteine was added together with the other unlabelled components. Zinc-treated samples were obtained by adding 10 µM $ZnSO_4$ to the expression medium immediately after transfection. Cu(II)-treated cells were obtained by adding 100 µM $CuCl_2$ 48 hours after transfection, followed by 24 h treatment prior to cell collection. To monitor DJ-1 oxidation, cells expressing DJ-1 were treated with 10 mM H_2O_2 for 1 hour before cell collection.

In-cell NMR sample preparation

Samples for in-cell NMR were prepared following a reported protocol [33]. Transfected cells were detached with trypsin-EDTA 0.05%, suspended in DMEM + 10% FBS, washed once with PBS and re-suspended in one pellet volume of DMEM supplemented with 90 mM glucose, 70 mM HEPES and 20% D_2O. The cell suspension was transferred in a 3 mm Shigemi NMR tube, which was gently spun to sediment the cells. Cell viability before and after NMR experiments was assessed by trypan blue staining. After the NMR experiments, the cells were collected and the supernatant was checked for protein leakage by NMR. The cell lysates were prepared by freeze-thaw cycles in PBS buffer followed by centrifugation to remove the insoluble fraction.

Expression and purification of DJ-1

E. Coli BL21(DE3) Gold cells (Stratagene) were transformed with the plasmid “DJ-1 TEV site pET15b”. Cells were grown overnight at 37°C in LB, harvested and re-suspended in ^{15}N-labelled M9 medium. Protein expression was induced with 0.5 mM IPTG and carried on at 37 °C; the cells were harvested after 4 h from

induction. Protein purification was performed using a nickel chelating HisTrap 5 ml column (GE Healthcare). After digestion with AcTEV protease (Invitrogen) overnight at 25 °C the protein was separated from the affinity tag in the HisTrap column. Finally, the protein was exchanged against in 20 mM KPi buffer pH 7, and 5 mM DTT was added to prevent protein aggregation.

Metal binding and oxidation of DJ-1 in vitro

Zinc-DJ-1 and Cu(II)-DJ-1 were prepared by titrating aliquots of [U-^{15}N]-DJ-1 with up to 1 equivalent-per-monomer of $ZnSO_4$ and $CuCl_2$, respectively, in KPi 20 mM buffer, pH 7. The spectral changes were monitored by NMR. Cu(I)-DJ-1 was prepared in an oxygen-free glove box, by titrating [U-^{15}N]-DJ-1 (in oxygen-free buffer) with up to 0.5 equivalents-per-monomer of a 50 mM stock solution tetrakis(acetonitrile)copper(I) hexafluorophophate, $[Cu(I)(CH_3CN)_4]PF_6$, dissolved in acetonitrile. The spectral changes were monitored by NMR. Oxidized DJ-1 was obtained as follows: an aliquot containing 100 µM DJ-1 was washed with 20 mM KPi buffer pH 7 in a 10 kDa-cutoff centricon to remove DTT and subsequently incubated with 1 mM H_2O_2 overnight at 4°C. Excess of unreacted H_2O_2 was removed by washes in centricon.

NMR data collection and analysis

In-cell NMR data were collected at a 950 MHz Bruker Avance spectrometer equipped with a TCI CryoProbe. For each cell sample, 1D ^{1}H and ^{1}H-^{15}N SOFAST-HMQC [35] spectra were recorded at 308 K (total acquisition time ~1 h). The same spectra were recorded on the corresponding cell lysates. *In vitro* NMR data were collected on samples of purified DJ-1 at either a 950 MHz or a 900 MHz Bruker Avance spectrometer, both equipped with TCI CryoProbes. ^{1}H-^{15}N SOFAST-HMQC spectra of apo DJ-1, Cu(II)-DJ-1 and oxidized DJ-1 were acquired at 308 K; ^{1}H-^{15}N SOFAST-HMQC spectra of zinc-DJ-1 and Cu(I)-DJ1 were acquired at 298 K. The spectra were processed with Topspin NMR data processing software and analyzed with CARA. The previously reported backbone amide resonance assignment of DJ-1 [36] (BMRB Entry 17507) was transferred to the ^{1}H-^{15}N NMR spectra of reduced and oxidized apo DJ-1 at 298 K with the aid of 3D NOESY-HSQC spectra, and subsequently transferred to the spectra recorded at 308 K, by

collecting a series of spectra at increasing temperatures. Combined Chemical Shift Difference (CCSD) values for each amide signal were calculated with the formula: $CCSD = \sqrt{\frac{(\Delta\delta\ ^1H)^2 + (\Delta\delta^{15}N/5)^2}{2}}$.

Mass spectrometry

ElectroSpray Ionization-Mass Spectrometry (ESI-MS) analysis was performed with a Thermo LTQ Orbitrap ($FT\text{-}MS^n$) spectrometer on unlabelled samples of 37 µM reduced and oxidized DJ-1. Both samples were dialyzed against MilliQ H_2O prior to analysis.

Results

Intracellular DJ-1 is soluble and free from interactions

In order to determine its intracellular conformation in the absence of external stimuli, DJ-1 was overexpressed in HEK293T cells in [U-^{15}N]-growth medium, and analyzed by in-cell NMR. The resulting ^{1}H-^{15}N NMR spectra were of surprisingly high quality considering the relatively high molecular size of the DJ-1 dimer (Figure 1a). Most signals arising from DJ-1 could be resolved in the spectra, indicating that the protein does not interact with slowly-tumbling cellular components and behaves as a free solute in the cytoplasm. Comparison with *in vitro* NMR spectra (Figure 1c) revealed that, in untreated cells, intracellular DJ-1 is dimeric and metal-free, with C106 in the reduced state. Upon cell lysis, a spectrum virtually identical to that recorded on DJ-1 *in vitro* was obtained (Figure 1b). Minor chemical shift differences were observed between *in vitro* and in-cell (Figure 1d) and lysate resonances (Figure 1e), which are likely due to small differences in pH, ionic strength and to small, weakly-interacting co-solutes.

DJ-1 does not bind divalent metal ions in the cell

DJ-1 has been shown to bind zinc and copper ions *in vitro* [26–28]. To determine the metal binding behavior of the protein in the cellular environment, we analyzed the conformation of DJ-1 in ^{1}H-^{15}N NMR spectra recorded on cells treated with zinc or copper in excess. The in-cell NMR spectra of zinc-treated cells did not reveal any appreciable changes in chemical shift or intensity of DJ-1 resonances with respect to untreated

cells, indicating that no metal binding occurred in the cell (Figure 2a,c). Comparison with the NMR spectra of [U-^{15}N]-DJ-1 titrated *in vitro* with zinc confirmed this hypothesis: zinc binding induced clear chemical shift changes in the proximity of the metal binding site previously identified (Figure 2b,d). Also in the case of Cu(II) treatment, no changes in chemical shift or intensity were observed with respect to untreated cells (Figure 2e,g), whereas *in vitro* titration of DJ-1 with Cu(II) caused the complete disappearance of the residues close to the binding site, and severe broadening of farther residues, due to paramagnetic relaxation enhancement (Figure 2f,h). Comparison of the signal intensity ratios (Figure 2g,h) clearly shows that no paramagnetic effect is observed in cells, indicating that intracellular Cu(II) binding does not occur.

DJ-1 does not promote Cu(I) transfer to SOD1

Treatment of cultured human cells with Cu(II) is expected to increase the intracellular availability of Cu(I), in accordance with the known pathway of copper uptake in eukaryotes [37]. Consistently, we investigated also the Cu(I) binding properties of DJ-1 in Cu(II)-treated cells. Cu(I) binding *in vitro* induced subtle but clearly visible changes in the ^{1}H-^{15}N NMR spectra of DJ-1: few signals arising from C53 and from residues structurally adjacent to it decreased in intensity, possibly broadened by chemical exchange, while the other protein signals were unperturbed (Figure 3a). In Cu(II)-treated cells, the corresponding signals of DJ-1 did not decrease in intensity (Figure 2e), suggesting that DJ-1 did not bind Cu(I) at the C53 binding site.

The strict cellular control over copper trafficking is mediated by specific metallochaperones that allow Cu(I) to reach its final acceptors [38]. We have previously shown that Cu(I) delivery to intracellular SOD1 can be promoted by co-expressing the copper chaperone CCS [32, 39]. Therefore, if DJ-1 acted as an alternative copper chaperone for SOD1 as suggested, one would expect that co-expression of DJ-1 and SOD1 in the presence of sufficient levels of both zinc and copper (as SOD1 needs to bind zinc to fold correctly) would lead to the observation of fully mature SOD1 (Cu,Zn-SOD1), mediated by a transient Cu(I)-DJ-1 species. However, this was not the case, as copper-free SOD1 was still mostly detected in the imino region of the 1D ^{1}H in-cell NMR spectra, essentially as much as a control sample in which DJ-1 was not co-expressed (Figure 3b). A sample of cells in which DJ-1 and CCS were co-expressed in the presence of copper was also analyzed, to assess whether CCS could deliver Cu(I) to DJ-1. Also in this case, the in-cell NMR spectra did

not reveal any significant changes compared to untreated cells, set aside for few additional signals belonging to CCS (Figure 3c).

Cysteine 106 is quantitatively oxidized to sulfinic acid

The thiol group of C106 in the active site of DJ-1 is known to be sensitive to oxidation in cells exposed to oxidative stress, however multiple oxidation states have been reported. We therefore analyzed the conformation of intracellular DJ-1 in cells treated with H_2O_2, and compared it with *in vitro* samples at defined C106 oxidation states. The in-cell NMR data revealed that DJ-1 in cells treated with 10 mM H_2O_2 for 1 h is quantitatively oxidized, resulting in a single species being produced (Figure 4a). The same species could be reproduced *in vitro* by incubating DJ-1 with 1 mM H_2O_2 overnight at 4 °C (Figure 4b), as shown by chemical shift analysis (Figure 4d,e). ESI-MS analysis of unlabelled protein samples identically prepared indicated that upon treatment *in vitro* with H_2O_2, reduced DJ-1 (Figure 4f) was oxidized to sulfinic acid (C106–SO_2H, Figure 4g), and that the reaction did not proceed to sulfonic acid (C106-SO_3H). To determine whether other oxidation states were produced in H_2O_2-treated cells, lysates from [^{15}N]-cysteine labeled cells were analyzed. The resulting ^{1}H-^{15}N NMR spectra confirmed that a single species (C106–SO_2H) was produced following treatment with H_2O_2 , as no additional signals arising from C106 in other redox sates of were present (Figure 4c).

Discussion

The proposed metallochaperone function of DJ-1 has joined an already long list of biological roles that have been attributed to this ubiquitous protein [17]. The metal binding properties of DJ-1 have been characterized *in vitro*, however, direct proof of protein metallation *in situ* has been lacking. By in-cell NMR, changes in protein conformation occurring in cells can be directly monitored at high resolution, making it an ideal approach to validate functional hypotheses in the physiological environment. Overall, our data indicate that in our experimental conditions DJ-1 does not bind intracellular zinc or copper ions, despite both having been supplemented in excess and being incorporated in the cells, as previously verified in the same conditions [32, 40]. This outcome can be explained by considering that the intracellular concentration of free metal, both

copper and zinc, is kept negligible by the cellular homeostasis [41]. For the most part, these metals are bound to other molecules either involved in metal trafficking or being the final acceptors. In the case of zinc, the amount of metal *available* for binding to any given protein depends roughly on the binding affinity of the latter compared to that of all the other competitors. For zinc, most intracellular protein binding sites (zinc fingers and metallothioneins being the most abundant) have affinities estimated in the picomolar range (K_d = 10^{-11} to 10^{-12} M [42]), while the intracellular glutathione ($K_d = 6.3 \times 10^{-5}$ M) does not act directly as a zinc pool [42]. In addition, active transport systems shuttle the excess zinc to zinc storing-organelles or remove it from the cell through a process called 'muffling' [43]. Given that the reported affinity of DJ-1 for zinc ($K_d = 6 \times 10^{-7}$ M [26]) is relatively low, one can expect the amount of zinc available to DJ-1 to be negligible, even in cells treated with excess zinc. This outcome is opposite to that of SOD1, which has a much higher affinity ($K_d = 4.2 \times 10^{-14}$ M [44]) and spontaneously binds zinc in the same cellular environment [32].

Copper trafficking is even more strictly regulated, and copper transfer only occurs via associative ligand substitution between copper-binding proteins [37]. Specific copper delivery pathways exist for each of the few cellular copper proteins, in which metallochaperones relay copper ions from the plasma membrane transporter CTR1, which imports copper as Cu(I), to each final acceptor in a process driven by increasing affinities for copper [38, 45]. Such specific pathways also act as kinetic barriers, preventing unwanted molecules from receiving copper. All known intracellular copper chaperones bind Cu(I) with high affinity, ranging between 10^{-14} and 10^{-16} M [45]. In this context, a physiological role for Cu(II) binding of DJ-1 seems unlikely. On the other hand, the reported affinity of the Cu(I)-binding site at the homodimer interface of DJ-1 ($K_d = 6 \times 10^{-16}$ M [28]) falls within the range expected for an intracellular copper protein. Despite this, in our experimental conditions, Cu(I)-DJ-1 was not formed, and co-expression of DJ-1 together with SOD1 did not promote the formation of Cu,Zn-SOD1, indicating that copper transfer mediated by DJ-1 did not occur. A possible explanation for this outcome is that DJ-1, like other copper proteins, needs a specific metallochaperone for its own maturation. Increased levels of the final acceptor (in this case, DJ-1) might overload the endogenous copper-delivery pathway, which has to be boosted by increasing the levels of metallochaperone, in analogy with SOD1 needing increased levels of CCS. Currently, however, no hypothesis has been made on the existence of such metallochaperone for DJ-1. The involvement of CCS in DJ-1 copper delivery can be excluded, as increasing CCS levels did not have any effect on DJ-1 metallation.

While the possibility that DJ-1 binds Cu(I) under certain conditions cannot be ruled out, these data show that DJ-1 cannot act as a copper chaperone itself, as unlike CCS it cannot receive Cu(I) directly from CTR1 at the plasma membrane.

DJ-1 is known to be oxidized in response to cellular oxidative stress. The oxidation is mediated by reactive oxygen species (ROS) and occurs selectively on the reactive C106 residue in the active site of the protein. Intracellular C106 oxidation has been reported to produce both cysteine-sulfinic (–SO_2H) and -sulfonic (–SO_3H) acid [20, 21]. While there is consensus that C106–SO_2H is the functionally relevant form, multiple oxidation states of C106 have been reported upon cell treatment with oxidizing agents [46, 47]. By in-cell NMR, the intracellular speciation of C106 could be directly accessed, as the chemical shift is highly sensitive to small changes in the chemical environment. In-cell NMR data revealed that H_2O_2-treated cells contain only one species of DJ-1, in which C106 is oxidized to sulfinic acid. [^{15}N]-cysteine labeling further reduces spectral complexity and simplifies data analysis, allowing multiple redox species, if present, to be identified [48, 49]. As no other species were detected in NMR spectra of [^{15}N]-cysteine labeled samples, it can be estimated that >90% of the total DJ-1 is in the C106-sulfinic acid form. Notably, these data were obtained with the highest concentration of H_2O_2 that preserved cell viability, and treating the cells with lower concentrations of H_2O_2 for the same period of time is unlikely to oxidize DJ-1 to a higher oxidation state. Therefore, in this cellular system, C106–SO_3H does not form upon acute treatment with H_2O_2. These results are consistent with the reactivity of C106 *in vitro*, where C106–SO_2H forms readily, sometimes spontaneously during sample handling [20], while oxidation to C106–SO_3H requires harsher conditions [10]. Nevertheless, the fact that C106–SO_3H DJ-1 has been observed under certain circumstances [21, 46] suggests that its formation might occur in other types of cells, or under stress conditions closer to the protracted, low-level stress occurring in dopaminergic neurons.

Conclusions

By using a direct, high resolution approach, we have assessed the biological relevance of several post-translational modifications occurring to DJ-1 that have been linked to severe pathologies such as Parkinson's

Disease, Amyotrophic Lateral Sclerosis and cancer. The results obtained suggest that the metal binding properties of DJ-1 active site (i.e. E18 and C106) towards copper and zinc should be reconsidered in light of the low binding affinity. While thermodynamically allowed, Cu(I) binding to the C53 residues at the dimer interface does not occur, indicating that DJ-1 cannot autonomously act as a copper chaperone.

Furthermore, we have shown that the oxidation state of C106 of intracellular DJ-1 can be monitored directly by in-cell NMR. Oxidation of C106 to sulfinic acid occurs efficiently in H_2O_2-treated cells, and the reaction does not proceed further to sulfonic acid. With such approach, it will be possible to determine how DJ-1 responds to varying concentration of oxidant and to other types of cellular stress, such as treatment with rotenone. Moreover, the time dependence of the intracellular oxidation process could in principle be studied by real-time in-cell NMR spectroscopy.

In addition to specific conformational changes, in-cell NMR also reveals that the protein is free from any interaction with intracellular partners. In our experience, this behavior is somewhat unexpected when considering the many DJ-1 cellular functions. Indeed, a protein with chaperone activity and regulation roles in many pathways (such as transcriptional regulation, proteasome inhibition) would more likely be invisible by in-cell NMR due to the plethora of interacting partners found in the cell. An example of the such behavior is given by profilin, which is simultaneously interacting with actin, phosphoinositides and several other proteins [50]. In comparison, an enzymatic activity of DJ-1 such as protease, glyoxalase or deglycase would be more consistent with the observed behavior, as the intracellular efficiency of an enzyme depends on its ability to diffuse freely within a cellular compartment, reaching its substrates where their concentration is higher. In this respect, being able to observe structural features of intracellular DJ-1 makes possible to further investigate the enzymatic activity of DJ-1 through in-cell NMR, especially in light of its recently reported activity as protein and DNA deglycase [15, 16].

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Figures

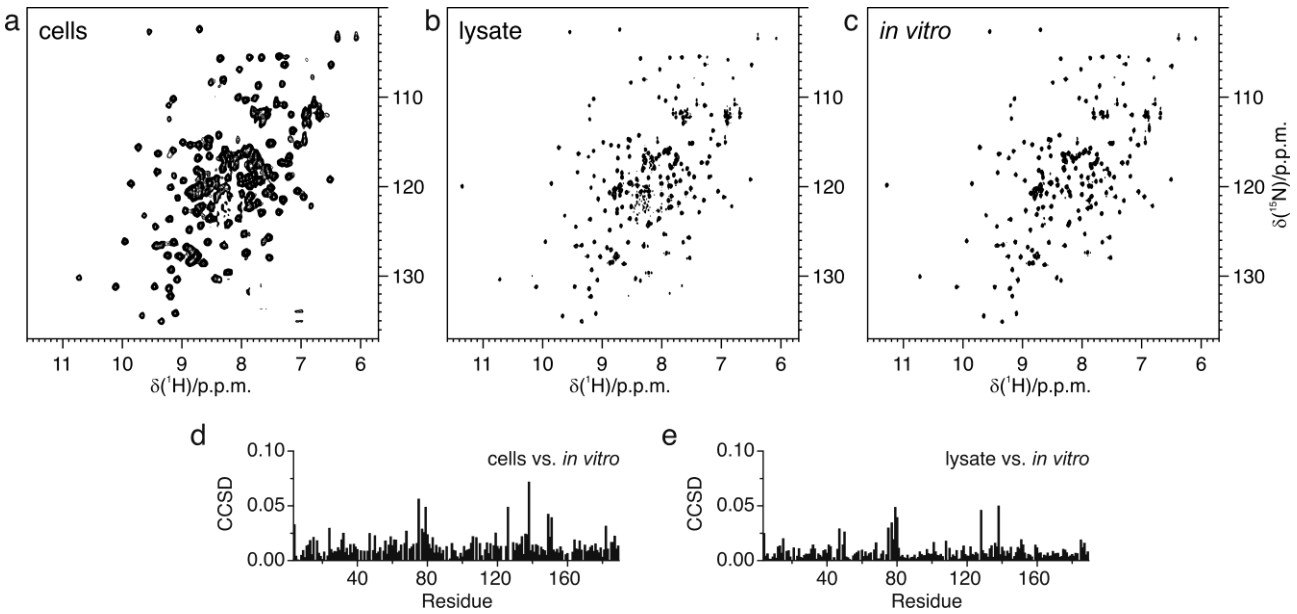

Figure 1. (a) ^{1}H-^{15}N NMR spectrum of cells expressing [U-^{15}N]-DJ-1; (b) ^{1}H-^{15}N NMR spectrum of the corresponding lysate; (c) ^{1}H-^{15}N NMR spectrum of purified apo, reduced [U-^{15}N]-DJ-1; (d) CCSD calculated for each residue between DJ-1 in-cell and *in vitro*; (e) CCSD calculated for each residue between DJ-1 in the cell lysate and *in vitro*.

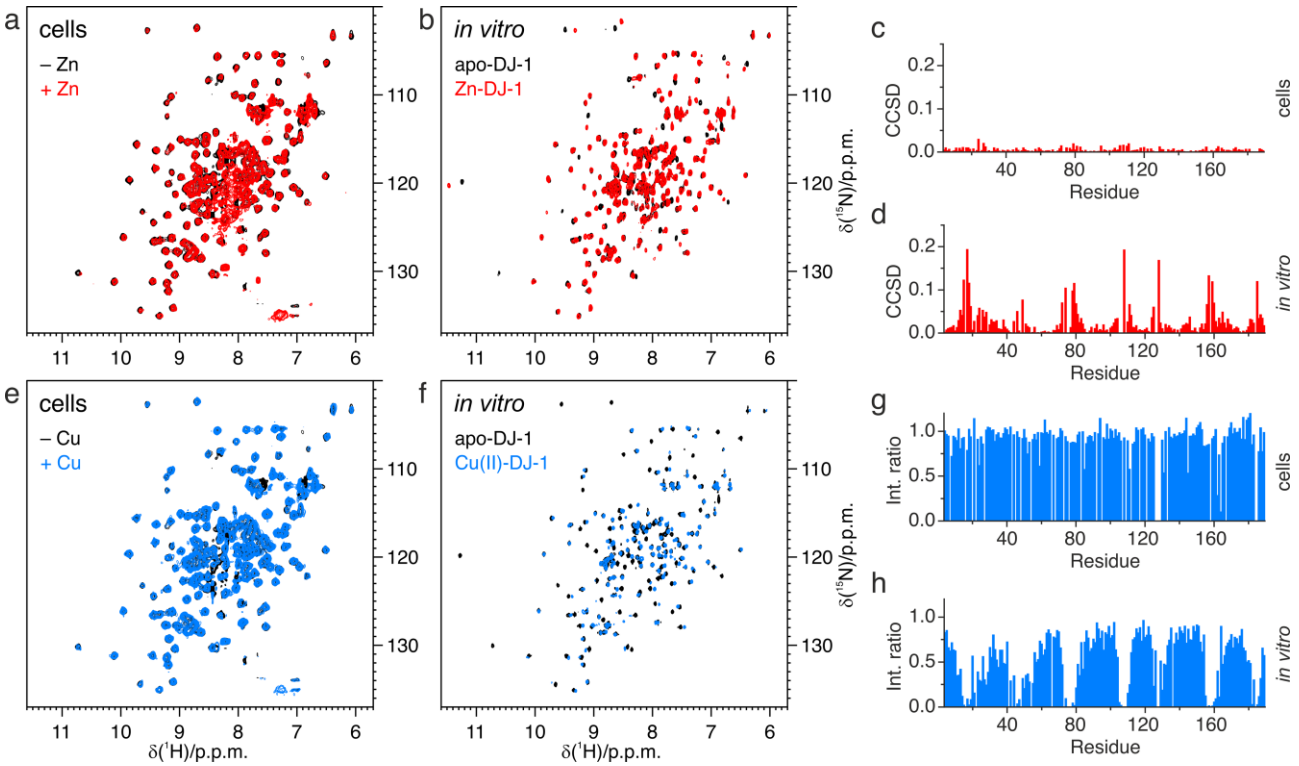

Figure 2. (a) ^{1}H-^{15}N NMR spectra of cells expressing [U-^{15}N]-DJ-1 in the absence (black) or in the presence (red) of excess zinc; (b) ^{1}H-^{15}N NMR spectra of purified apo-DJ-1 (black) and Zn-DJ-1 (red); (c) CCSD plot between DJ-1 in zinc-treated cells and DJ-1 in untreated cells; (d) CCSD plot between Zn-DJ-1 and apo-DJ-1 *in vitro*; (e) ^{1}H-^{15}N NMR spectra of cells expressing [U-^{15}N]-DJ-1 either untreated (black) or treated (blue) with excess copper; (f) ^{1}H-^{15}N NMR spectra of purified apo-DJ-1 (black) and Cu(II)-DJ-1 (blue); (g) signal intensity ratio calculated for each amide signal between DJ-1 in copper-treated and DJ-1 in untreated cells; (h) signal intensity ratio between Cu(II)-DJ-1 and apo-DJ-1 *in vitro*.

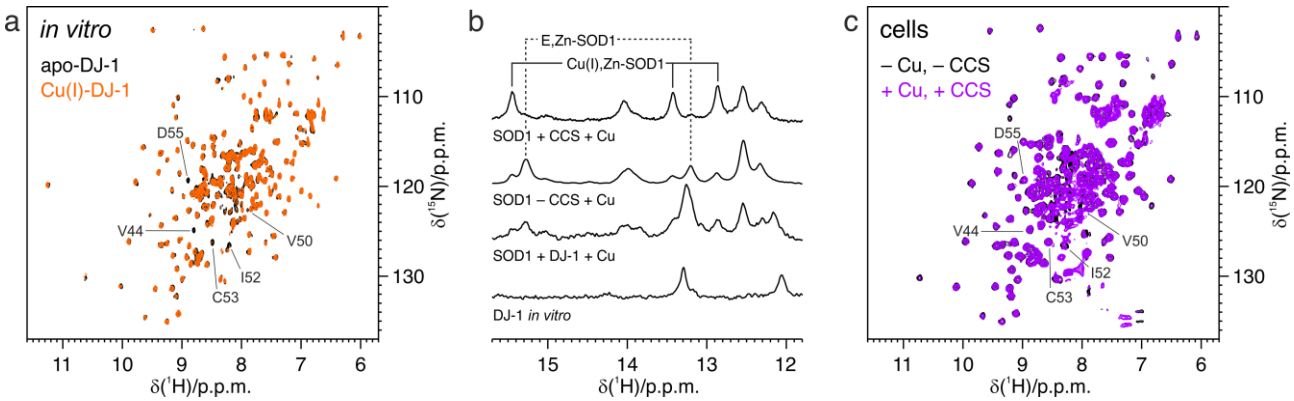

Figure 3. (a) ^{1}H-^{15}N NMR spectra of purified apo-DJ-1 (black) and Cu(I)-DJ-1 (orange). The signals disappearing upon Cu(I) binding are labeled; (b) the imino region of ^{1}H NMR spectra acquired, from top to bottom, on copper-treated cells co-expressing SOD1 and CCS, on copper-treated cells expressing SOD1 alone, on copper-treated cells co-expressing SOD1 and DJ-1, on purified DJ-1 *in vitro*. Histidine signals from E,Zn-SOD1 and Cu(I),Zn-SOD1 are indicated; (c) ^{1}H-^{15}N NMR spectra of cells expressing DJ-1 in the absence of copper (black) or co-expressing DJ-1 and CCS in the presence of copper (violet). The signals which disappear upon Cu(I) binding *in vitro* are labeled.

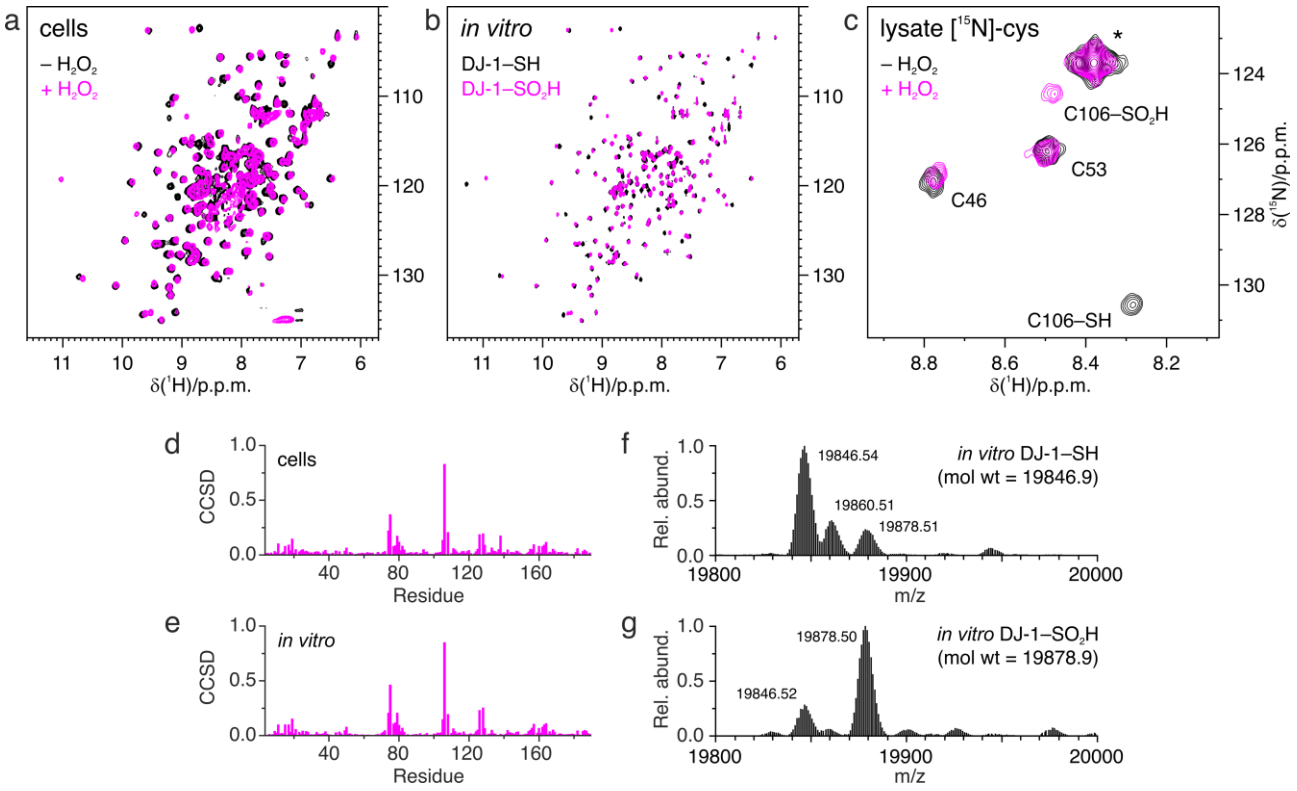

Figure 4. (a) ^{1}H-^{15}N NMR spectra of cells expressing [U-^{15}N]-DJ-1 without (black) or with (magenta) treatment with H_2O_2; (b) ^{1}H-^{15}N NMR spectra of purified reduced DJ-1 (DJ-1–SH, black) and oxidized DJ1 (DJ-1–SO_2H, magenta); (c) ^{1}H-^{15}N NMR spectra lysates from cells expressing [^{15}N-cysteine]-DJ-1 without (black) or with (magenta) treatment with H_2O_2. The signals arising from DJ-1 cysteines are labeled, and the oxidation states of C106 as determined *in vitro* are indicated; (d) CCSD plot of DJ-1 between H_2O_2-treated and untreated cells; (e) CCSD plot between DJ-1–SO_2H and DJ-1–SH *in vitro*; (f) ESI-MS spectrum of purified DJ-1–SH; (g) ESI-MS spectrum of purified DJ-1–SO_2H.