Disulfide-mediated stabilization of DJ-1, a protein implicated in Parkinson disease

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By
Brian A. Williams

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ABSTRACT

DJ-1, a protein that causes recessive early-onset Parkinson disease (PD), is believed to be destabilized in PD. Covalent dimerization of DJ-1, with cyclic disulfides, is a potential strategy to stabilize the protein while restoring its native homodimeric form. Here we attempt to elucidate more information about the underlying details of covalent dimerization, including its specificity. We also examine the effect of non-cyclic disulfide mediating binding to DJ-1 on the protein’s stability and investigate its specificity. We discover multiple methods of stabilizing DJ-1 selectively: either through covalent dimerization at Cys53 using a cyclic disulfide or covalent modification at Cys106 using a non-cyclic disulfide.
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INTRODUCTION

Parkinson disease (PD) is a neurodegenerative disease that includes symptoms such as tremor, rigidity, and bradykinesia. It is also characterized histopathologically by the presence of Lewy bodies\(^1\). These Lewy body’s fibrils are rich in the proteins ubiquitin and α-synuclein. Autosomal recessive mutations in the gene PARK7, encoding the protein DJ-1, are a cause of early-onset familial PD\(^2\). DJ-1 is a member of the ThiJ/Php family of proteins, predicted to have a similar active site and function as chaperone protein YajL\(^3\). Though the precise biochemical function of DJ-1 is unknown, it has been found that DJ-1 binds RNA\(^4\), associates with transcription factors\(^5\), undergoes SUMOylation to promote cell growth\(^6\), protects dopaminergic neurons against rotenone-induced apoptosis\(^7\), and reduces oxidative stress in cells\(^8\). DJ-1 is primarily found in astrocytes and glial cells, with a smaller amount found in neurons\(^9\). It has also been reported to form complexes with, and ameliorate the toxicity of, mutant Cu/Zn superoxide dismutase, a cause of familial amyotrophic lateral sclerosis (ALS)\(^10\).

While the destabilizing mutations L166P, A104T, and M26I are associated with familial PD, over-oxidized DJ-1 is found in sporadic PD and this over-oxidation also destabilizes DJ-1. Therefore, a generic therapeutic strategy against PD could be to find a way to reduce the over-oxidation of DJ-1. There exists no treatment that prevents progression of the disease, so more potential therapeutic pathways are needed. Dimer stabilization of DJ-1 may be another therapeutic strategy for PD. Stabilization of dimeric DJ-1 may restore a loss-of-function caused by PD-associated mutations and modifications, such as the ability to reduce oxidative stress in cells\(^11\). Specifically, stabilizing the dimer near the dimer interface at Cys53 has been a strategy
we have pursued. Cyclic disulfide molecules, capable of undergoing thiol-disulfide exchange, have been identified by our lab as molecules that can facilitate the formation of covalent dimers of WT DJ-1. In particular, the cyclic disulfide 1-oxo-1,2-dithiane (NSC56224), was found to bind to Cys53’s, one on each monomer, to “covalently dimerize” WT DJ-1. Of note, while NSC56224 was able to form substantial amounts of covalently dimerized DJ-1, neither the analogous dioxide (1,1-dioxide-1,2-dithiane; NSC62632), nor the closely related 4,5-dihydroxy-1,2-dithiane (oxidized DTT, NSC663605) (Figure 1), were very reactive in being able to form covalent dimers, suggesting an important role for the sulfoxide oxygen of NSC56224 in this two-step (unmodified monomer -&gt; NSC56224-modified monomer -&gt; NSC56224-tethered dimer, Figure 2) reaction.

In particular, the NSC56224-modified monomer (i.e. pre-covalent dimerization DJ-1) was not observed in LC-MS data, suggesting the initial cyclic disulfide binding to one monomer is rate-limiting, and leaving it uncertain whether the sulfoxide oxygen was indeed involved in either step of the reaction. To further elucidate the mechanism of NSC56224-mediated covalent
dimerization of DJ-1, we sought to determine 1) whether loss of oxygen occurred prior to or during the first or second binding step of the covalent dimerization reaction and 2) whether the observed specificity for cyclic disulfides at Cys53 was due to the ability of cyclic disulfides to act reversibly at single cysteines (via thiol-disulfide exchange) and irreversibly at cysteine pairs (due to loss of reversibility following entropically favorable interactions at appropriately placed cysteines).

To determine the sequence of binding intermediates, reactions between NSC56224 and the moderately dimer destabilized A104T DJ-1 were performed. The reduced dimer propensity for A104T DJ-1 was allowed us to detect the intermediate NSC56224-modified monomer that was absent in prior experiments performed using WT DJ-1. To investigate the mechanism of cyclic disulfide-mediated specificity of modifying DJ-1 at Cys53, non-cyclic disulfides were used to probe the reactivity of the surface cysteines of DJ-1 for thiol-disulfide exchange in the absence of the self-reversibility provided by the cyclic structure of 1-oxo-1,2-dithiane. This was of particular interest as DJ-1 includes the highly reactive and functionally crucial Cys106, and highly reactive dopamine quinones, capable of covalently dimerizing DJ-1, were shown to non-selectively modify both Cys53 and Cys106\(^\text{12}\). Indeed, non-cyclic disulfides identified that modify DJ-1 appear to favor reaction with Cys106 – an important observation given that this residue is the primary site of oxidation in vivo. Interestingly, non-cyclic disulfide mediated modification of DJ-1 (without covalent dimerization) was sufficient to stabilize DJ-1. Given that one of the original strategies for pharmacological chaperone-mediated protein stabilization was to bind the active site, this did not come as a surprise. Disulfide-mediated modification of
Cys106 suggests a potential endogenous mechanism that could regulate DJ-1 function, which might go unobserved by common protein analytical methods that utilize reductants.

**EXPERIMENTAL SECTION**

**Expression and purification of DJ-1**

Expression and purification of WT, A104T, C53S, and C106S DJ-1 was done similarly to previous literature. Rosetta strains of E. coli were transformed with a pET-15b expression vector containing the DJ-1 expression construct, then grown to an OD$_{600}$= 0.6 in LB broth, and supplemented with 100 µg/mL ampicillin at 37°C. Then overexpression was induced upon the addition of 0.5 mM IPTG, where it was incubated for an additional 3 hours at 37°C. The cells were then harvested by centrifugation and stored at -80°C until purification began. Purification began once frozen cells were thawed, resuspended in 50mM Tris/2 mM EDTA/ 1 mM DTT (pH 7.4) and lysed by sonication. The crude cell extract was cleared by centrifugation at 13,000 rpm and ammonium sulfate was added to 70% saturation to the supernatant, which was then incubated at 4°C for 30 minutes, after which centrifugation followed. The supernatant was loaded onto a phenyl sepharose column equilibrated for 70% ammonium sulfate and 10 mM potassium phosphate (pH 6.8). Protein was eluted with decreasing ammonium sulfate gradient to 10 mM potassium phosphate (pH 6.8) over 10 column volumes. Fractions containing DJ-1 were identified using MALDI-TOF-MS, pooled and dialyzed overnight against 1 mM potassium phosphate. The sample was loaded onto a Mono Q column equilibrated with 10 mM potassium phosphate, 70% ammonium sulfate and 1 mM NaCl and eluted with a 10-column volume gradient from 10 to 500 potassium phosphate (pH 6.8). Fractions containing DJ-1 were again identified with MALDI-TOF-MS, pooled buffer exchanged, and concentrated. Typical yields
were at ~1mg/L as determined by a Bradford protein assay. Purified proteins were aliquoted, stored at -80°C and thawed before use.

**Non-cyclic disulfide treatment**

Non-cyclic disulfides were acquired from the National Cancer Institute/ DTP Open Chemical Repository, Bethesda, MD, USA) and dissolved to 100 mM in DMSO, aliquoted and stored at -80°C. Proteins and compounds were further diluted in 10 mM Tris-HCl, pH 7.4 to necessary concentrations before the reactions were run. The non-cyclic disulfide incubation was performed with compounds in 1000X molar excess and incubated for 24 hours. NSC 54088 (2,2'-dithiodiacetic acid, Figure 3) was identified to bind to DJ-1 and was then rerun at 10X molar excess with the protein and analyzed with Liquid Chromatography-nanoelectrospray ionization-Quadrupole Ion Trap-Mass Spectrometry (LC-ESI-IonTrap-MS).

![Figure 3. NSC 54088](image)

**Cyclic disulfide treatment of A104T DJ-1**

Similarly to the previous covalent dimerization reported and the screen of non-cyclic disulfides, PD mutant A104T was diluted and incubated for 24 hours with 10X molar excess NSC 56224 which were then analyzed with LC-ESI-IonTrap-MS.

**LC-ESI-IonTrap-MS analysis**

1 picomole each of treated and untreated DJ-1 that was incubated with the various non-cyclic disulfide compounds were analyzed by LC-NanoESI-IonTrap-MS. Samples were loaded onto a packed C18 column (5 μm TARGA, Higgins Analytical, Mountain View, California, USA) and eluted over a gradient (utilizing buffers A and B as HPLC-grade water with 0.1% formic acid
and HPLC-grade acetonitrile with 0.1% formic acid) consisting of: 5% B for 7 minutes, 5-50% B for 36 minutes, 50-95% B for 4 minutes, 95% B for 8 minutes, and 95-5% B for 16 minutes. Mass spectra were acquired using an HCTultra PTM Discovery System IonTrap (Bruker Daltonics, Billerica, Massachusetts, USA). The data were then analyzed using DataAnalysis 3.4 (Bruker Daltonics, Billerica, Massachusetts, USA). Spectra were acquired from retention times where DJ-1 eluted and were deconvoluted using Maximum Entropy calculations (Spectrum Square Associates, Inc., Ithaca, NY, USA). Incubations with each compound and subsequent analyses were performed in duplicate.

**Differential Scanning Fluorimetry (DSF)**

To determine the effect on thermally stabilizing DJ-1 of the various binding compounds differential scanning fluorimetry was performed\(^{14}\). WT and C53S DJ-1 (20 µM in 10mM Tris-HCl, pH 7.4) were incubated with 1, 5, and 25X molar excess of NSC 54088 for 24 hours at room temperature in a 96 well reaction plate (MicroAmp Fast Optical, Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA). After 24 hour incubation, SYPRO Orange (Life Technologies Corporation, Carlsbad, California, USA), a fluorescent dye that binds to exposed aromatic residues, was added to the mixture to a concentration of 20X, and the reaction was then centrifuged. The fluorescence from each well of the plate was monitored using an RT-PCR machine (Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA); which was run using a temperature gradient of 25 to 99.9°C. Samples were repeated in triplicate. Curve fitting was performed in MATLAB. Statistical significance of changes in melting temperature were evaluated by Kruskal-Wallis one-way ANOVA followed by Dunn’s post-hoc analysis.
RESULTS

NSC56224-modified A104T DJ-1 monomer reveals oxygen is present on the cyclic disulfide, and its loss as water promotes the second thiol-disulfide exchange and covalent dimerization.

Given that the A104T mutant form of the protein maintains the overall structure of DJ-1 we hypothesized that it could also be covalently dimerized. It, in fact, was covalently dimerized during its incubation with NSC 56224 (Figure 4).

![Figure 4. A104T covalent dimerization](image)

The covalent dimerization of A104T may provide insight into why NSC 56224 is such a good molecule to promote the thiol-disulfide exchange reaction because in the covalently dimerized spectrum there is still some monomer present with a +16 fragment, indicating oxygen is bound to the monomer.
NSC 54088, a non-cyclic disulfide, modifies WT DJ-1

Non-cyclic disulfides were incubated with DJ-1 and then analyzed with LC-ESI-IonTrap-MS.

The molecule with the greatest amount of binding to WT DJ-1 was NSC 54088 (Figure 5).

![Figure 5. MS Spectra of WT DJ-1 + NSC 54088](image)

The deconvoluted mass spectra are shown in figure 5. No dimerization of DJ-1 was found in the non-cyclic disulfide incubation indicating covalent dimerization did not occur with the molecule, increasing the likelihood that these non-cyclic disulfides may react at the more reactive cysteine 106 instead of Cys53. Whereas Cys 53 is located at the subunit interface and is close enough to its two-fold related counterpart to form a cross-link between the monomers, Cys 106 is located far from the interface and cannot mediate covalent reaction with its counterpart.

NSC 54088 enhances the thermal stability of DJ-1 in a Cys53-independent manner
Seeing that covalent dimerization of WT DJ-1 with a cyclic disulfide enhances the thermal stability of DJ-1, the thermal stability of DJ-1 after its reaction with NSC 54088 was also examined. Denaturation of NSC 54088 treated and untreated DJ-1 was induced using increasing temperature through DSF. As previously stated WT and C53S DJ-1 were incubated with 1, 5, 25X molar excess NSC 54088 for 24 hours at room temperature and analyzed. For both WT and C53S DJ-1 there was concentration-dependent increase in the calculated denaturation temperature of DJ-1 with $\Delta_{\text{melting temperature}}$ as great as 5.8 ± 0.26°C for WT and 3.8 ± 0.11°C for C53S (±SD., n = 3, Figures 6 and 7)

![Denaturation Temperatures](image)

**Figure 6.** Denaturation temperatures of WT DJ-1 + non-cyclic and cyclic disulfides
Figure 7. Denaturation temperatures of C53S DJ-1 + non-cyclic and cyclic disulfides

This indicates Cys53 is not affected in binding of DJ-1 by non-cyclic disulfide-mediated modification.
**DISCUSSION**

Covalent dimerization has been suggested as a potential therapeutic agent for SOD-1 because of the assumed role of monomerization of this protein in amyotrophic lateral sclerosis\(^{15}\). Cross-linkers have been found for SOD-1 in recent literature\(^{16}\). Similarly to SOD-1, DJ-1 contains surface cysteines which can be utilized to covalently dimerize DJ-1. Recent literature has shown positive results for finding DJ-1 binding compounds as a result of *in silico* methods to identify potential compounds capable of modifying its oxidative state\(^{17}\). These compounds bind, in vivo, to Cys106, the cysteine known to be overly oxidized in PD\(^{17b, 18}\). This Cys106 residue is known for its ability to form sulfinic acids by being oxidized, to an extent which is neuroprotective but that can be deleterious if oxidation is excessive\(^{19}\).

**Potential mechanisms of covalent dimerization and modification**

Covalent dimerization and simple modification at Cys53 and Cys106, respectively, are interesting chemically given the differences in mechanisms (Figures 8 and 9).

![Figure 8. General scheme of covalent dimerization of DJ-1 at Cys 53](image)

![Figure 9. General scheme of covalent modification of DJ-1 at Cys 106](image)
The molecules themselves may provide even more insight into the mechanisms of these reactions. The oxygen on NSC 56224 likely acts as an oxidant as it leaves the compound, making the second step of the reaction more favorable. This further enhances the irreversibility of the reaction (Figure 2). Interestingly, similar compounds, which were also included in the cyclic disulfide screen (NSC 62632 and NSC 663605), did not produce any covalent dimerization of WT DJ-1. This is likely because NSC 56224 reaches a certain “sweet spot” in terms of molecular reactivity. NSC 62632 contains two oxygen atoms bound to one of the sulfurs in the compound; therefore there may be steric and electronic hindrance preventing the second step of the reaction from occurring. Alternatively, while removal of a single oxygen from sulfenic acid (by reduction, for example) is often possible, removal of the second and third oxygen from sulfinic and sulfonic acid is often not possible. On the other hand, NSC 663605 does not have any other groups besides the disulfide that make the reaction favorable for covalent dimerization. So, the oxygen on NSC 56224, which can act as an oxidant and leaving group, apparently makes this reaction extremely favorable.

This theory that the oxygen of NSC 56224 promotes covalent dimerization is also evidenced in the covalent dimerization of A104T DJ-1 (Figure 3). Given that A104T is less stable than wild-type, the overall reaction is slower. We can then see some monomer bound with NSC 56224, which still contains the oxygen bound, whereas the dimer formed, which now includes NSC 56224 without the oxygen bound to the molecule. This covalent dimerization provides some insight to this mechanism, indicating that the oxygen leaving step is a necessary part of the reaction mechanism after the initial thiol-disulfide exchange occurs.

**NSC54088 mediated covalent modification occurs at Cys 106 in the active site of DJ-1**
On the other hand, it seems that non-cyclic disulfides do not follow the same reaction mechanism nor bind at the same cysteine as cyclic disulfides. In the mass spectra of NSC 54088 binding to WT DJ-1 we see DJ-1 plus half (91 m/z) the molecular mass of the compound (full molecular mass 182 m/z) indicating our reaction scheme, where the disulfide bond is broken and the molecule splits, to be correct (Figure 9). As previously mentioned, Cys106 is the more reactive surface cysteine so it would be expected that any thiol-disulfide exchange reaction would occur there as opposed to cysteine 53. Therefore, given that we found that WT and C53S DJ-1 is stabilized by NSC 54088, while NSC 56224 did not stabilize C53S, we can safely assume that it is binding and modifying DJ-1 at a different site, and this site is likely Cys106. There would seem to be no advantage for the non-cyclic disulfide to bind at Cys53, so this conclusion seems plausible.

While it is entropically favorable for cyclic disulfides to covalently dimerize DJ-1 at Cys53 (the second step benefits from a chelate effect) it is just as energetically favorable for non-cyclic disulfides to modify DJ-1 at Cys106. There would be no energetic gain if the cyclic disulfide bound to Cys106, as the oxygen would not be able to leave the molecule. Given that Cys106 is amenable to oxidation, the thiol-disulfide exchange that likely occurs with NSC 54088 is more favorable, at that site than a reaction at Cys53. Specifically, the reaction of covalent dimerization likely occurs as shown in figure 1 while the mechanism of non-cyclic disulfide modification occurs as drawn in the general scheme given our data.

Non-cyclic disulfides thus indicate another way of stabilizing DJ-1. Given that Cys106 is the proposed active site of DJ-1, covalently modifying this residue with a non-cyclic disulfide may have the biological effect of making it an inhibitor of DJ-1. This may not be therapeutically relevant but from a structural biology perspective this is an interesting idea. This can potentially
allow for NSC 54088 to be used as a chemical probe of DJ-1’s activity. One might be able to tag a sulfur or carbon to then follow the protein’s movements and reactions. This could also help to further elucidate the reaction mechanism of its covalent modification and can be done to the cyclic disulfides as well.

PD-mutants instabilities

Among the most studied PD associated mutations of DJ-1 are L166P, A104T, and M26I. The L166P variant is massively destabilized, with severely reduced dimer propensity\textsuperscript{20}. This leads to the idea that the point mutation destabilizes the tertiary and quaternary structure, causing loss of homodimerization and in turn loss of function. The A104T and M26I mutations are also destabilizing but they do not disrupt dimer formation as severely as the L166P mutation (although a reduced capacity to form homodimers has been observed in cells\textsuperscript{21}). A104T DJ-1 has been found to induce PD heterozygously while M26I and L166P DJ-1-associated PD are the results of homozygous mutations\textsuperscript{22}. DJ-1’s ability to reduce oxidative stress is also affected by the L166P mutation. L166P DJ-1 was shown to be ineffective in protecting cells from oxidation by H\textsubscript{2}O\textsubscript{2} compared to WT\textsuperscript{23}. It will be of great interest to attempt to covalently dimerize and/or modify the PD-mutants of DJ-1 and investigate their stabilities, and multiple PD-associated variants have been purified for that purpose.

Conclusion

Two selective methods (each targeting different cysteine residues) of modification and stabilization of DJ-1 were found using cyclic and non-cyclic disulfides. These methods employ different mechanisms and give us insight into the protein’s structure and function. Covalent dimerization at Cys53 may be a therapeutic strategy to stabilize DJ-1, thereby increasing the
steady-state level of this neuroprotective protein in the cell. On the other hand, the non-cyclic disulfide modification and stabilization of DJ-1 may at the very least enable us to learn more about DJ-1’s functions in vivo.

REFERENCES


