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Dityrosine Cross-links are Present in Alzheimer’s Disease-derived Tau Oligomers and Paired Helical Filaments (PHF) which Promotes the Stability of the PHF-core Tau (297–391) In Vitro


1 - Sussex Neuroscience, School of Life Sciences, University of Sussex UK
2 - Biomedical Science Research and Training Centre, Yobe State University, Nigeria
3 - Chemistry Department, College of Sciences, Mustansiriya University, Baghdad, Iraq
4 - Analytical Development Biologics, Biopharmaceutical Development, Syngene International Limited, Biocon Park, Bommasandra Jigani Link Road, Bangalore 560009, India
5 - Institute of Medical Sciences, University of Aberdeen, UK
6 - TauRx Therapeutics Ltd, Aberdeen, UK

Correspondence to Louise C. Serpell: L.C.Serpell@sussex.ac.uk (L.C. Serpell) mahmoudbukar (M.B. Maina), @serpellab, @serpell1 (L.C. Serpell)
https://doi.org/10.1016/j.jmb.2022.167785
Edited by Daniel Otzen

Abstract

A characteristic hallmark of Alzheimer’s Disease (AD) is the pathological aggregation and deposition of tau into paired helical filaments (PHF) in neurofibrillary tangles (NFTs). Oxidative stress is an early event during AD pathogenesis and is associated with tau-mediated AD pathology. Oxidative environments can result in the formation of covalent dityrosine crosslinks that can increase protein stability and insolubility. Dityrosine cross-linking has been shown in Aβ plaques in AD and α-synuclein aggregates in Lewy bodies in ex vivo tissue sections, and this modification may increase the insolubility of these aggregates and their resistance to degradation. Using the PHF-core tau fragment (residues 297 – 391) as a model, we have previously demonstrated that dityrosine formation traps tau assemblies to reduce further elongation. However, it is unknown whether dityrosine crosslinks are found in tau deposits in vivo in AD and its relevance to disease mechanism is unclear. Here, using transmission electron microscope (TEM) double immunogold-labelling, we reveal that neurofibrillary NFTs in AD are heavily decorated with dityrosine crosslinks alongside tau. Single immunogold-labelling TEM and fluorescence spectroscopy revealed the presence of dityrosine on AD brain-derived tau oligomers and fibrils. Using the tau (297–391) PHF-core fragment as a model, we further showed that prefibrillar tau species are more amenable to dityrosine crosslinking than tau fibrils. Dityrosine formation results in heat and SDS stability of oxidised prefibrillar and fibrillar tau assemblies. This finding has implications for understanding the mechanism governing the insolubility and toxicity of tau assemblies in vivo.

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Introduction

Tau protein was first described as a cytoplasmic protein that plays a role in microtubule dynamics.\(^1\)\(^-\)\(^2\) However, recent evidence suggests that it plays multiple functions, including DNA protection, heterochromatin stability and nucleolar transcription.\(^3\)\(^-\)\(^6\) Interest in tau intensified following the discovery that it is the main component of the intracellular neurofibrillary tangles (NFTs), comprised of straight (SFs) and paired-helical filaments (PHFs), which forms one of the hallmarks of Alzheimer’s disease (AD), alongside extracellular deposits of amyloid-beta (A\(\beta\)).\(^7\)\(^-\)\(^10\) Under native conditions, tau is soluble and intrinsically disordered. However, post-translational modifications, truncation, mutations or cellular stress conditions dered. Nonetheless, dityrosine formation results in heat increase filament stability\(^29\) similar to stability and insolubility of brain-derived PHFs.\(^30\)\(^-\)\(^32\)

Given the high stability and irreversibility of the dityrosine covalent bonding,\(^33\) crosslinked proteins can be detected in various in vitro and in vivo settings. A\(\beta\) and \(\alpha\)-synuclein have both been shown to form dityrosine cross-links in vitro, and dityrosine cross-linked A\(\beta\) and \(\alpha\)-synuclein have each been localised in A\(\beta\) plaques in AD and in Lewy bodies in Parkinson’s disease (PD) post-mortem brain tissues, respectively.\(^34\)\(^-\)\(^37\) This raises the question of whether dityrosine cross-linking is present on human-derived tau assemblies. Using TEM immunogold labelling and fluorescence spectroscopy, we show that dityrosine cross-linking is observed on human AD tau oligomers and PHFs, and dityrosine colocalises with tau on NFTs in AD. However, using MCO of dGAE in vitro, we showed that prefibrillar tau assemblies are more amenable to dityrosine cross-linking than fibrils, suggesting that dityrosine crosslinking in vivo may have a different impact on the tau assemblies and activity. Nonetheless, dityrosine formation results in heat and SDS stability of oxidised prefibrillar and fibrillar tau assemblies, suggesting a general role of dityrosine crosslinking in enhancing pathological tau stability.

Results

Dityrosine crosslinking is observed in pathological tau from AD samples

We have previously shown the presence of dityrosine crosslinks in AD amyloid plaques.\(^36\) Here, we first conducted immunogold labelling TEM on AD brain sections to investigate the presence of dityrosine crosslinks on pathological tau. The labeling of AD brain sections revealed densely packed tau-containing filaments, many showing the characteristic paired-helical appearance upon close inspection (Figure 1(A)). These filaments also labelled with an anti-dityrosine antibody, indicating the presence of dityrosine crosslinks. Since PHFs were not found in the age-matched control brain sections, only the data from the AD patients is shown in Figure 1(A). Very low immunogold labeling levels were observed for age matched controls (Figure S2).

Ex-vivo derived AD tau filaments\(^25\) were also examined using immunogold TEM. Electron micrographs show the labelling of anti-dityrosine on the tau filaments supporting the view that the tau protein is cross-linked within the filaments (Figure 1(B)). To examine whether dityrosine crosslinks occur earlier in the assembly process, AD brain-derived oligomers\(^38\)\(^-\)\(^39\) were labelled with dityrosine antibody using single immunogold labelling, and these show the distinctive gold labels suggesting that dityrosine is also found within these earlier oli-
In support of the presence of dityrosine crosslinks within brain derived oligomeric tau, a fluorescence emission peak was observed for the oligomeric species in solution at 410 nm, characteristic of the presence of dityrosine (Figure 1(C)). Together, these data reveal that dityrosine crosslinks appear in both tau oligomers and fibrils in vivo and ex-vivo, suggesting a potentially important role for dityrosine in tau assembly and neurofibrillary tangle accumulation in AD.

**Soluble and prefibrillar dGAE are more amenable to dityrosine crosslinking and its impact than fibrillar dGAE assemblies**

To further examine the potential effects of dityrosine crosslinking on fibrillar tau, we examined the labelling of filaments formed from dGAE, which has been shown to form PHFs and SFs without additives.\(^24,26-27,41-42\) Agitation of 300 \(\mu\)M dGAE for 6 h at 37°C C resulted in the formation of a mixture of oligomeric and pre-fibrillar species, while incubation for 48 h resulted in mature filaments with a pronounced twist and some lateral bundling (Figure 2(A), (B), (C)). We have shown that metal-catalysed oxidation (MCO) using Cu\(^{2+}\) and H\(_2\)O\(_2\) results in dityrosine crosslinking of the dGAE.\(^23\) In vivo, reactive oxygen species are produced from hydrogen peroxide catalysed by a metal ion such as Cu\(^{2+}\) via the Fenton reaction and Harber-Weiss cycle. This results in production of tyrosyl radical formation, isomerisation and finally enolation.\(^34\) In this study, soluble, prefibrillar and fibrillar dGAE samples (50 \(\mu\)M) were prepared from their respective stock preparations (Figure 2). The 50 \(\mu\)M soluble, pre-fibrillar and fibrillar forms of dGAE were incubated for 15 mins or 48 h under MCO conditions using Cu\(^{2+}\) and H\(_2\)O\(_2\) to induce the formation of dityrosine crosslinks. The tyrosine and dityrosine levels within the samples were evaluated together by fluorescence
spectroscopy using an excitation wavelength of 280 nm for tyrosine and excitation of 310 nm for dityrosine (Figure 3(A)-(D)). A small peak is observed at 360 nm which arises due to a Raman scattering effect from the water in phosphate buffer. Soluble dGAE shows a strong tyrosine fluorescence signal which is reduced substantially following a 15 min incubation in oxidising conditions (Figure 3(A)), with the concurrent increase in the dityrosine signal at 410–420 nm (Figure 3(B)). Similarly, prefibrils showed a transition to dityrosine fluorescence following oxidation (Figure 3(A)-(B)).

The level of dityrosine signal in the soluble and prefibrillar dGAE samples remained similar between 15 min and 48 h suggesting that the formation of dityrosine is rapid (Figure 3(B) & (D)). In contrast, mature fibrillar dGAE showed an extremely low dityrosine signal at 15 min, which remained similar at 48 h (Figure (B) & (D)), suggesting that fibrils are less susceptible to dityrosine crosslinking. The tyrosine signal decreases further from 15 min to 48 h incubation for all samples. For unoxidized samples, this may be due to lateral association of fibrils with time leading to the burial of the tyrosine. Indeed, tyrosine signal for unoxidised fibrils remains constant between 15 min and 48 h time point. For oxidised samples, this is due to a transition to dityrosine. Together, this reveals that soluble and prefibrillar dGAE assemblies are more amenable to dityrosine crosslinking than fibrillar species.

To investigate the secondary structure of the assemblies, circular dichroism spectroscopy (CD) was conducted (Figure 3(H)-(J)). CD spectra for fibril samples show the expected β-sheet signal for the unoxidised fibril sample which increases with time, but the decreased intensity β-sheet signal for the oxidised sample may a further decrease in solubility upon oxidation. This seems to suggest that changes happen rapidly and there is little further conformational change after 15 mins incubation following oxidation and dityrosine crosslinking.

The appearance of soluble, prefibrillar and fibrillar samples of dGAE that had been oxidised for 15 mins only or remained unoxidised were compared using TEM. TEM is qualitative method rather than quantitative and images shown are representative of what was observed on the grids. As expected, the electron micrographs of unoxidised dGAE
samples showed very small particles for soluble dGAE, short fibrils for prefibrillar and longer fibrils for those categorised as fibrils (Figure 4(A)-(F)). In contrast soluble (Figure A and B) and prefibrillar (Figure C and D) samples appeared to form small and larger rounded structures following oxidising incubation, suggesting that the oxidation of amino acid residues, including tyrosine to form dityrosine resulted in the prevention of further elongation and formation of trapped, relatively stable spherical species. However, the fibrillar sample remains fibrillar and their morphology changed very little compared to the unoxidised samples. Some lateral association of fibrils was apparent, and this may arise from crosslinking between fibres (Figure E and F).

After 48 h incubation, all three samples incubated under unoxidised conditions had assembled to some extent with either very long fibrils, or shorter fibres in the soluble dGAE sample (Figure 4 E-F).
In comparison, after incubation in oxidising conditions, the soluble and prefibrillar samples showed mostly trapped, spherical species (similar to those found at 15 mins incubation) but with some short fibrillar protofibrils (narrower elongated fibrils). The fibrillar sample showed shortening of the fibrils, suggestive of fragmentation or breakage of the stabilised fibres, or prevention of elongation, with more obvious lateral association than observed after 15 mins incubation. This may indicate that these cross-linked stabilised fibres are susceptible to breakage during incubation with shaking.

To investigate the apparent stability caused by the dityrosine crosslinking further, we investigated the heat and SDS insolubility of the oxidised and unoxidised samples (Figure 5(A)). The samples were boiled at 100 °C in Laemmli sample buffer containing 4.4% LDS and 10% β-mercaptoethanol and then resolved by SDS–PAGE electrophoresis and stained with Coomassie brilliant blue. Unoxidised soluble dGAE sample mainly separated as a monomer (10/12 kDa) while prefibrillar and fibrillar samples show the appearance of a dimer (20 kDa/24 kDa). Insoluble species are found in the well and are most clearly evident in the fibril sample as expected. For the oxidised samples, there is a clear increase in the protein material in the well for each soluble, prefibrillar and fibrillar sample compared to the unoxidised and a dimer appears in all three samples. The fibril sample also shows some higher molecular weight species which suggests the stabilisation of larger species potentially via dityrosine as well as other surface interactions. The structure of paired helical filaments consists of two protofilaments with two tau molecules associated so that tyrosines can participate in dityrosine on both sides of the filament. Furthermore, each protofilament contains multiple tyrosines.

This shows that 15 mins oxidation alone results in heat and SDS insolubility of the soluble, prefibrillar and fibrillar assemblies. This is more pronounced in the fibrillar sample, which indicated that heating and incubation in the Laemmli buffer resulted in the dissociation of the majority of the unoxidised fibrils to monomers and multimers. However, the oxidised samples that were treated the same way showed assemblies that remained as monomers, multimers, and some were too large and would have failed to enter the gel (≥250 kDa, red arrows). This suggests that the oxidised assemblies maybe stabilised through dityrosine formation.

Comparison of heat and SDS denatured (fully denatured) with SDS only (partially denatured) showed that non-oxidised fibrils run as a dimer with staining in the well from insoluble (presumably fibrillar) material, while the fully denatured condition leads to monomer with low well staining (Figure S3). In contrast, while oxidised fibrils also run as dimer and monomer in partially and fully denatured respectively, they show staining in the well under both conditions and the monomer and dimer bands are weak intensity. This supports the view that oxidation leads to increased resistance to denaturation.

dGAE contains a single cysteine residue at residue 322. To examine whether the oxidation of cysteine plays a role in the conformational and morphological changes in dGAE upon oxidation, the ability of C322A variant dGAE to form dityrosine was investigated and compared with a control dGAE with a Y310A substitution. Oxidised wildtype dGAE shows a strong signal from dityrosine at 410-420 nm as previously shown, and this remains clearly observed for C322A dGAE with a similar intensity as wildtype (Figure 5(B)-(C)). As expected, however, this signal is not observed for Y310F since this variant does not include a tyrosine residue. ThS fluorescence shows that wildtype dGAE and C322A dGAE assemble over 48 h only when incubated under non-oxidising conditions, while Y310F appears to be unable to self-assemble under either oxidising or non-oxidising conditions used in this study (Figure 5(E)-(G)). Collectively these data showed that cysteine is not involved in the dGAE stabilisation following oxidation, while tyrosine appears to be involved in dGAE assembly.

**Discussion**

Dityrosine occurs naturally in proteins, helping to increase their stability. This modification has been found in many elastic and structural proteins, including elastin, fibroin, keratin, cuticlin, and...
collagen,43–46 where it is believed to increase their mechanical strength and subsequently their insolvability.47 However, the formation of dityrosine in proteins is also implicated in many diseases, including AD, and PD,36–37,40,48 cystic fibrosis,49 atherosclerosis,50 cataracts in the eye lens 51–52 and acute myocardial infarction.53 Our previous work has co-localised dityrosine with Aβ within Aβ plaques36 and Lewy bodies with α-synuclein,35,37 suggesting that it could play a role in increasing the insolubility of Aβ plaques in AD and α-synuclein aggregates in PD. It remained to be determined whether dityrosine crosslinking is formed in the tau aggregates in human AD brains. Here, we have revealed that dityrosine forms in vivo on tau oligomers and filaments within NFTs both in situ in brain and also localised to ex-vivo AD-derived assemblies. Using the PHF-core tau fragment (dGAE) as a model, which forms dityrosine cross-links via tyrosine 310, we also showed that although dityrosine forms on tau aggregates, mature fibrillar species are less amenable to dityrosine crosslinking and that dityrosine formation promotes heat and SDS insolubility of the tau aggregates.

It is known that exposure to reactive oxygen species (ROS), ageing, metal ions and other factors could induce dityrosine crosslinking on the tau oligomers and fibrils in vivo. Dityrosine crosslinking on tau fibrils could increase their stability and insolubility. The PHFs extracted from the AD brain are highly insoluble and resistant to proteolytic cleavage. Specifically, the early-stage PHF-tau has reduced SDS solubility,31,59 while late-stage PHF tau exhibits SDS and sarcosyl insolubility.30–32 Previous work on full-length tau aggregation with arachidonic acid showed that dityrosine crosslinked tau fibrils have increased SDS stability, suggesting that the crosslinking may play a role in stabilising the early-stage PHFs and confers stability to convert into late-stage PHFs.29 Here, we find that the oxidised dGAE fibrils are difficult to resuspend in solution, are heat and SDS-insoluble and remained strongly laterally associated to one another, shown by TEM. Given that we find dityrosine crosslinking on AD-derived tau fibrils and in NFTs, this finding suggests that this post-translational modification increases the insolubility of tau fibrils and NFTs in AD.

By comparing the capability of soluble, prefibrillar and fibrillar dGAE to form dityrosine, our findings indicate that fibrillar tau assemblies are less amenable to dityrosine crosslinking. This is evidenced by the very low fluorescence signal observed in the oxidised fibrillar dGAE samples in

**Figure 5. Stability of crosslinked assemblies, dityrosine formation susceptibility and self-assembly in dGAE variants.** Soluble, prefibrils and fibrils of dGAE (50 µM) were prepared from their respective stock assemblies, left unoxidised or oxidised using CuCl2:dGAE at 1:10 ratio, then mixed with 2.5 mM H2O2 and left for 15 min at 37 °C/700 rpm. Compared to all unoxidised assemblies, the oxidised assemblies showed heat and SDS insolubility that is more pronounced in the fibril sample (A). 50 µM soluble wild-type dGAE, dGAE with C322A substitution and dGAE with Y310F substitution were prepared, left unoxidised or oxidised using CuCl2:dGAE at 10:1 ratio then mixed with 2.5 mM H2O2 and left for 15 min or 48 h at 37 °C/700 rpm. Fluorescence readings showed an increase in intensity signal for dityrosine in oxidised wildtype dGAE (B) and C322A dGAE (C) samples at 15 min and 48 h. No dityrosine signal was detected in Y310F dGAE sample (D). Unoxidised wildtype dGAE (E) and C322A dGAE (F) assemblies incubated for 48 h showed increased ThS fluorescence suggesting assembly, while Y310F dGAE showed no self-assembly (G). In contrast, oxidised samples showed no ThS fluorescence suggesting prevention of assembly.
spectroscopic experiments, unlike in the oxidised soluble and prefibrillar assemblies, which showed robust dityrosine formation. This apparent inability to form dityrosine compared to soluble and prefibrillar assemblies could be due to the inaccessibility to the tyrosine residue on the tau molecule when it is arranged in a fibrillar architecture. The only tyrosine present in dGAE is at position 310, which is buried within the PHF core in the Cryo-EM structures of tau filaments from many tauopathies, including AD and CTE. Therefore, it can be concluded that the decreased ability of dGAE fibrils to form dityrosine under MCO conditions is due to the inaccessibility of the tyrosine residue, unlike in the soluble and prefibrillar dGAE assemblies.

Our in vitro work on dGAE and Aβ both suggest that dityrosine formation traps the assemblies in a conformation that doesn’t favour further elongation into fibrils. However, whether dityrosine crosslinked tau oligomers also fail to elongate to fibrils in vivo is a question of future research. We have shown that dityrosine crosslinked tau oligomers are not toxic to human neuroblastoma cells even after three days in culture, although we also find that dGAE is generally non-toxic in its oligomeric form. We have also shown that dityrosine does appear to be associated with neurofibrillary tangle in AD tissue and extracted species. However, the stage at which dityrosine formation occurs during the assembly and deposition of tau remains to be elucidated. It is unclear at the moment what the impact of dityrosine crosslinking is on the aggregation and toxicity of tau oligomers in vivo. Nonetheless, it is essential to highlight that the influence of dityrosine on tau aggregation and toxicity may depend on the extent of the dityrosine crosslinking. A highly crosslinked protein may be highly trapped and exhibit a different property than a partially crosslinked protein. This, therefore, makes it impossible to speculate a general model of the influence of this modification on tau and indeed other proteins like Aβ.

Finally, cysteine oxidation can be induced by oxidative stress conditions and is known to influence the functions, properties and toxicity of tau. dGAE has a single cysteine residue at position 322, and we have previously shown that it forms PHFs independent of this cysteine and that assembly is enhanced under reducing conditions or using C322A variant. To examine whether the oxidation of cysteine plays a role in the conformational and morphological changes in dGAE upon oxidation, we utilised dGAE C322A. This revealed that cysteine oxidation is not involved in stabilising the tau aggregates; instead, the effects observed resulted from dityrosine crosslinking. However, this does not rule out the involvement of other oxidative modifications, such as the oxidation of histidine and phenylalanine, which are all present in dGAE. Future studies would delineate the specific amino acids involved. Nonetheless, the immunogold labelling experiments on AD brain tissue and AD-derived fibrils and oligomers strongly suggest that dityrosine crosslinking has in vivo relevance to tau in AD, similar to its role on Aβ and α-synuclein in AD and PD, respectively. Given the role of dityrosine in increasing the mechanical strength and insolubility of proteins, we believe that its association with hallmarks of AD (tau and Aβ) and PD (α-synuclein) that are known to be insoluble in these diseases, could suggest its participation in promoting the insolubility, accumulation and resistance to degradation of NFTs and Aβ plaques in AD and α-synuclein aggregates in Lewy bodies in PD.

In conclusion, for the first time, our findings showed that, similar to Aβ plaques and α-synuclein Lewy bodies, dityrosine crosslinks PHFs in AD and is a posttranslational modification of AD-derived tau oligomers and fibrils. Our finding suggests that the assembly state of the tau molecule will influence its ability to be crosslinked, such that, when the tyrosine is buried in the case of fibrils, it becomes less amenable to dityrosine formation. This finding has implications for understanding the mechanism governing the insolubility and toxicity of tau assemblies in vivo and providing therapeutic avenues that focus on the tau molecule.

Materials and Methods

Cases

Tissue from the middle--frontal gyrus of three AD subjects and one age-matched control were used for transmission electron microscope (TEM) immunogold labelling. The samples were obtained from the London Neurodegenerative Disease Brain Bank with informed consent and under material transfer agreement and stored at −80 ℃ under local ethics committee guidelines (Table 1).

Immunogold labelling for total tau and dityrosine

The brain sections were labelled using our established method of immunogold labelling. Phosphate-buffered saline (pH 8.2) containing 1% BSA, 500 µl/l Tween-20, 10 mM Na EDTA, and 0.2 g/l NaN3 (henceforth called PBS+), was used throughout. Briefly, thin brain sections were mounted on TEM grids and blocked for 30 minutes in normal goat serum (1:10 dilution), labelled with 10 µg/ml IgG anti-tau rabbit polyclonal antibody (Sigma, SAB4501831) and 10 µg/ml IgG anti-dityrosine mouse monoclonal antibody (JaICA, Shizuoka, Japan), and left in a humid container overnight at room temperature. The sections were rinsed in PBS + three times for 2 minutes and
following heat treatment. The protein fractions were identified by P11 phosphocellulose chromatography followed by 50 mM PIPES buffer (pH 6.8) or 50 mM MES buffer (pH 6.25), both supplemented with 1 mM EGTA, 5 mM EDTA, 0.2 mM MgCl₂ and 5 mM 2-mercaptoethanol containing 0.1–1 M KCl. The peak of protein elution was identified by protein assay (at 0.3–0.5 M KCl) and dialyzed against 80 mM PIPES buffer (pH 6.8), 1 mM EGTA, 5 mM EDTA, 0.2 mM MgCl₂, 5 mM 2-mercaptoethanol, or PB (10 mM; pH 7.4). The dGAE protein concentration was measured using Advanced Protein Assay Reagent (Cytoskeleton, Inc.) with bovine serum albumin as a standard. dGAE was finally diluted with 10 mM phosphate buffer (pH 7.4). dGAE (300 μM) was prepared in phosphate buffer (10 mM; pH 7.4) and incubated in the dark for i) 0 minutes; ii) 6 hours; or iii) 48 hours at 37 °C with shaking at 700 rpm using a thermomixer C (Eppendorf, Germany). Based on TEM observations, henceforth, the samples will be referred to as (i) soluble dGAE; (ii) prefibrillar dGAE; and (iii) fibrillar dGAE, respectively.

Metal-catalysed oxidation of dGAE

The soluble, prefibrillar and fibrillar dGAE samples were diluted to 50 μM before being used for metal-catalysed oxidation. For the fibrillar samples, 50 μM of the fibrils were obtained by first determining the concentration of the fibrils. This was achieved by spinning the fibrillar dGAE for 30 minutes at 20,000 g to separate the supernatant (containing unaggregated, soluble dGAE) and pellet (containing fibrils). The concentration of the soluble dGAE in the supernatant was used to back-calculate the concentration of dGAE in the fibrils. Soluble, prefibrillar and fibrillar dGAE (at 50 μM) were incubated in phosphate buffer (10 mM; pH7.4) alone (unoxidised) or in the presence of 500 μM CuCl₂ (peptide: CuCl₂ ratio 1:10) and 2.5 mM H₂O₂ (oxidised) and incubated for 15 minutes or 48 hours at 37 °C with shaking at 700 rpm. The oxidation reaction was quenched by adding 2 mM EDTA to the assembly mixture. Oxidation of variant dGAE (carrying the mutations C322A and Y310F) was done using the same procedure. A minimum of three independent experiments were conducted to ensure the reproducibility of the findings.

Fluorescence spectroscopy

Dityrosine formation was monitored using a fluorescent excitation wavelength of 320 and emission collected between 340 – 600 nm, with
dityrosine peak signal expected between 400–420 nm, using a fluorescence spectrophotometer (Varian Ltd., Oxford, UK) and a 1-cm path length quartz cuvette (Starna, Essex, UK). The tyrosine fluorescence signal was monitored using an excitation wavelength of 280 nm and emission between 290 – 600, with the peak tyrosine emission observed at 305 nm. For all the measurements, the excitation and emission slits were set to 10 nm with a scan rate set to 300 nm/min with 2.5 nm data intervals and an averaging time of 0.5 s. The photomultiplier tube detector voltage was set at 500 V.

Thioflavin s (ThS) fluorescence assay

To assess the assembly of dGAE in the different samples, a portion (10 μL) of each was incubated for 3 min with ThS (5 μM in 20 mM MOPS buffer, pH 6.8). ThS fluorescence intensity was observed using a fluorescence spectrophotometer (Varian Ltd., Oxford, UK) and a 1-cm path length quartz cuvette (Starna, Essex, UK). The excitation wavelength was set at 440 nm, with emission between 460 to 600 nm collected, with the peak emission observed at 483 nm. Each experiment included a minimum of three independent experiments. The peak emission was collected and analysed, then plotted graphically.

Circular dichroism (CD)

The secondary structure of the samples was assessed using the Jasco J715 CD spectrometer (Jasco, Goh-Umstadt, Germany). Each sample (40 μL) was placed into a 0.2-mm path length quartz cuvette (Hellma) and scanned between 190 and 260 nm. The CD spectra were collected in triplicate at a temperature of 21 °C.

SDS-PAGE and immunoblotting

dGAE samples (20 μL) for each condition were mixed with 4x Laemmli sample buffer (containing 4.4% LDS) (Bio-Rad), supplemented with 10% 2-mercaptoethanol, boiled for 10 min at 100 °C, resolved using a 4%–20% gradient Mini-Protean TGX Precast Gels (Bio-Rad) at 100 V until the sample buffer reached the end of the gel. Partial denatured samples were mixed only with 4x Laemmli buffer but were not boiled and no 2-mercaptoethanol was used. The gels were stained using Coomassie Blue stain. Images were collected using LI-COR Odyssey XF.

Author contributions

M.B.M. planned and carried out the work; Y.K.A.-H., SO, TK, LB, KM and J.E.R. contributed experimental work. M.B.M. and L.C.S. wrote the paper; Y.K.A.-H and G.B. edited the paper and provided training. GB, CRH and CMW reviewed and edited the paper. LCS managed the project.

CRediT authorship contribution statement


Acknowledgements

The authors are grateful to M Goedert (MRC Laboratory of Molecular Biology, Cambridge, UK) for ex-vivo paired helical filaments which were kindly contributed by Bernandino Ghetti (Indiana University, USA). The authors are very grateful to Urmia Sengupta, Nemil Bhatt and Rakez Kayed (University of Texas, USA) for providing ex-vivo tau oligomers. TEM work was performed at the University of Sussex’s Electron microscopy imaging centre (EMC), funded by the School of Life Sciences, the Wellcome Trust (095605/Z/11/A, 208348/Z/17/Z) and the RM Phillips Trust. The authors thank Dr Pascale Schellenberger for valuable support.

Funding

This work was supported by funding from Alzheimer’s Society [AS-PG-16b-010] awarded to LCS and funding MBM. MBM is funded by the Alzheimer’s Association. YA is supported by WisTa Laboratories Ltd (PAR1596). The work was supported by ARUK South Coast Network. GB was supported by European Molecular Biology Organisation (EMBO) Short-Term Fellowship award (EMBO-STF 7674). LCS is supported by BBSRC [BB/S003657/1]. Urmia Sengupta, Nemil Bhatt, Rakez Kayed acknowledge the funding that supports their contribution (NIH grants to R.K: R01 AG054025 and U24AG072458).

Conflicts of Interest

C.R.H. and C.M.W. hold Offices within TauRx Therapeutics Ltd. and are named inventors on patents in the field of tau protein in neurodegenerative disorders. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data;
in the writing of the manuscript, and in the decision to publish the results. The remaining authors declare no conflicts of interest.

Data Sharing

No big data is generated from this work. The data generated and used to make figures in this manuscript will be available upon request.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2022.167785.

Received 29 May 2022; Accepted 3 August 2022; Available online 9 August 2022

Keywords:
Alzheimer’s disease; tau; dityrosine; paired helical filaments; oxidative stress

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