Doctoral Thesis

Reversible Electron Transfer from Tyrosine 125 to Oxidized Methionine 127 in α-Synuclein

Author(s):
Grandjean, Frédéric

Publication Date:
2015

Permanent Link:
https://doi.org/10.3929/ethz-a-010510904

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REVERSIBLE ELECTRON TRANSFER FROM TYROSINE 125 TO
OXIDIZED METHIONINE 127 IN α-SYNUCLEIN

A thesis submitted to attain the degree of DOCTOR OF SCIENCES of ETH ZURICH
(Dr. sc. ETH Zurich)

presented by

FREDERIC GRANDJEAN

MSc ETH Zürich

born on 06.05.1983 citizen of Juriens (VD)

accepted on the recommendation of

Prof. Dr. Willem H. Koppenol

Prof. Dr. Rudiyanto Gunawan

2015
ACKNOWLEDGEMENTS

This work would not have been possible without the help of many people. In particular, I would like to express my deepest gratitude to:

Prof. Dr. Willem Koppenol for providing me the opportunity to conduct research in his group and granting me freedom in defining and carrying out my PhD project, for contributing to the completion of my PhD project through his advice, and for contributing to improve my writing skills with his relevant advice.

All of the senior scientists of the Koppenol group:

Dr. Patricia L. Bounds for contributing to the orientation of my PhD project and for teaching me the essentials in scientific communication.

Dr. Anastasia Domazou for teaching me the essentials in kinetic analysis.

Dr. Thomas Nauser for teaching me the essentials in signal acquisition and kinetic analysis, for maintaining the group Febetron and for teaching how to use it to its fullest potential, and for contributing to the scientific quality of my PhD project through the invaluable discussions we had together.

Dr. Reinhard Kissner for teaching me the essentials in signal acquisition and electrochemistry, for maintaining the linear sweep voltammetry work station, for troubleshooting the imponderable and various troubles of a PhD project, and for contributing to the scientific quality of my PhD project through the invaluable discussions we had together.

I extend my special thanks to Prof. Dr. Roland Riek for allowing me to work in his laboratory and Dr. Silvia Campioni for teaching me the purification method of α-synuclein.

I am grateful to all the members of the Koppenol group whom I acquainted for turning the laboratory into a place of friendship and meaningful exchanges.

My heartfelt and deepest gratitude goes to my family for the unwavering support to me during my time as a PhD student in all the various forms that love inspires.
OBJECTIVES

The formation of stable nitrated oligomers of α-synuclein is widely acknowledged as a central factor in the etiology of Parkinson’s disease. These stable nitrated oligomers are stabilized by cross-linked o,o’-dityrosine. Therefore, the aim of this thesis is to characterize the behavior of the C-terminal domain of α-synuclein under oxidative conditions and to investigate the potency of monohydrogen ascorbate as a reducing reagent of the tyrosyl radicals of the tyrosine residues of α-synuclein. If monohydrogen ascorbate can reduce the tyrosyl radical in α-synuclein fast enough, this would hint at a potential role of monohydrogen ascorbate as a factor that prevents the onset of Parkinson’s disease.

Chapter 1 introduces Parkinson’s disease, the biology of α-synuclein, the biology and the chemistry of monohydrogen ascorbate, and why the oxidation of tyrosine 125 by oxidized methionine 127 is feasible. Chapter 2 describes the methods and experimental set ups used in this study. Chapter 3 describes the evidence for the reversible intramolecular electron transfer from tyrosine 125 to oxidized methionine 127 observed in the synthetic peptide Ac-AYEMPSE-NH$_2$, which corresponds to residues 124 to 130 of the α-synuclein C-terminal domain. Chapter 4 reports the results of the reaction rate of tyrosyl radical of α-synuclein with monohydrogen ascorbate. Finally, chapter 5 provides a conclusion and an outlook to this study.
# ABBREVIATIONS AND SYSTEMATIC NAMES

## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet and visible spectroscopy</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Wavelength in nm</td>
</tr>
<tr>
<td>Hasc$^-$</td>
<td>Monohydrogen ascorbate</td>
</tr>
<tr>
<td>Tyr/O$^*$</td>
<td>Tyrosyl radical</td>
</tr>
<tr>
<td>Met/S$^{+}$</td>
<td>Methionine sulfur radical cation</td>
</tr>
<tr>
<td>Met/S::X</td>
<td>Two center-three electron bond between sulfur and another atom</td>
</tr>
<tr>
<td>AE7</td>
<td>Synthetic peptide Ac-AYEMPSE-NH$_2$</td>
</tr>
<tr>
<td>AE7-M4A</td>
<td>Synthetic peptide Ac-AYEAPSE-NH$_2$</td>
</tr>
<tr>
<td>AE7-E3A</td>
<td>Synthetic peptide Ac-AYAMPSE-NH$_2$</td>
</tr>
<tr>
<td>AE7-P5A</td>
<td>Synthetic peptide Ac-AYEMASE-NH$_2$</td>
</tr>
<tr>
<td>EE7</td>
<td>Synthetic peptide Ac-EGYQDYE-NH$_2$</td>
</tr>
<tr>
<td>EA18</td>
<td>Synthetic peptide Ac-EAYEMPSEEGYQDYEPEA-NH$_2$</td>
</tr>
<tr>
<td>AE14</td>
<td>Synthetic peptide Ac-AYEMPSEEGYQDYE-NH$_2$</td>
</tr>
<tr>
<td>SE14</td>
<td>Synthetic peptide Ac-SYEDPPQEEYQEYE-NH$_2$</td>
</tr>
<tr>
<td>SNCA</td>
<td>Gene $\alpha$-synuclein</td>
</tr>
<tr>
<td>SYNA</td>
<td>Protein $\alpha$-synuclein</td>
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## Systematic names

<table>
<thead>
<tr>
<th>Compound</th>
<th>Systematic name</th>
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</thead>
<tbody>
<tr>
<td>Hydroxyl radical</td>
<td>Hydridooxygen(•)</td>
</tr>
<tr>
<td>Nitrogen monoxide</td>
<td>Oxonitrogen(•)</td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>Oxoperoxonitrate(1--)</td>
</tr>
<tr>
<td>Peroxonitrous acid</td>
<td>Hydrogen oxoperoxonitrate</td>
</tr>
<tr>
<td>Superoxide</td>
<td>Dioxide(•1--)</td>
</tr>
<tr>
<td>Azidyl radical</td>
<td>Trinitrogen(2N-N)(•)</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MPP⁺</td>
<td>1-Methyl-4-phenylpyridin-1-ium</td>
</tr>
<tr>
<td>Paraquat</td>
<td>1,1'-Dimethyl-4,4'-bipyridinium dichloride</td>
</tr>
<tr>
<td>Rotenone</td>
<td>(2R,6aS,12aS)-1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9-dimethoxychromeno[3,4-b] furo(2,3-h)chromen-6-one</td>
</tr>
<tr>
<td>L-dopa</td>
<td>(S)-2-amino-3-(3,4-dihydroxyphenyl) propanoic acid</td>
</tr>
<tr>
<td>Dopamine</td>
<td>4-(2-aminoethyl)benzene-1,2-diol</td>
</tr>
<tr>
<td>6-Hydroxy-dopamine</td>
<td>5-(2-Aminoethyl)benzene-1,2,4-triol</td>
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SUMMARY

Parkinson’s disease is a disease strongly linked with oxidative stress resulting from mitochondrial damage. Neurons have the highest concentrations of monohydrogen ascorbate in comparison to other body tissues. α-Synuclein is a protein central to the etiology of Parkinson’s disease and is a major component of intracellular inclusion bodies called Lewy bodies. α-Synuclein is a protein target of oxidative stress and forms stable nitratred dimers under oxidative conditions. These dimers are covalently cross-linked proteins via α,α’-dityrosine bonds. The C-terminal domain of α-synuclein has been linked with fibril formation triggered by Tyr125.

Our aim was two-fold. Our first aim was to investigate the behavior of the C-terminal domain under oxidative conditions. In order to do so, we performed experiments on synthetic peptides that correspond in length up to the last 124 to 140 amino acids of the sequence of the C-terminal domain of α-synuclein. Our second aim was to investigate whether the reducing agent monohydrogen ascorbate can reduce the tyrosyl radical faster than the tyrosyl radical in α-synuclein dimerizes. The tyrosyl radical was produced by the oxidation of tyrosine by trinitrogen(2N-N)(•).

We used pulse radiolysis to produce hydridooxygen(•) to make oxidative conditions. In the case of experiments with monohydrogen ascorbate, the sample solutions contained azide anions to scavenge hydridooxygen(•) and form trinitrogen(2N-N)(•). In the absence of tryptophan in the peptide or the protein investigated, trinitrogen(2N-N)(•) specifically targets tyrosine for oxidation.

We found that monohydrogen ascorbate reduces tyrosyl radical in α-synuclein faster than α-synuclein dimerizes. The rate constant of tyrosyl radical by monohydrogen ascorbate is \( k(\text{TyrO}^+ + \text{Hasc}^-) = 1.9 \pm 1.3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \) in the sample α-synuclein. The dimerization rate constant is \( k(\text{dimerization}) = 1.7 \pm 0.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1} \). If we take the lower end concentration of monohydrogen ascorbate in neurons [Hasc] = 2 mM, then the half-life of tyrosyl radical becomes 25 μs. The concentration of tyrosyl radical in α-synuclein necessary to obtain a dimerization half-life of 25 μs is 11 M. In this case, the ratio of tyrosyl radicals that undergo repair or dimerization is 50:50. If we assume that under extreme oxidative stress conditions, the
concentration of tyrosyl radical in α-synuclein is 1 µM, then within the first 25 µs, 0.5 µM of tyrosyl radical will be reduced by monohydrogen ascorbate whereas only 40 ppb of tyrosyl radical will dimerize. Therefore, we can confidently state that the reduction of tyrosyl radical in α-synuclein by 2 mM monohydrogen ascorbate excludes the possibility of dimerization of tyrosyl radical in α-synuclein.

Our second result is also of importance. Previous studies reported that oxidized methionine in the vicinity of tyrosine can oxidize tyrosine into a tyrosyl radical by means of an intramolecular electron transfer, possibly by tunneling. Not only did we observe the same intramolecular electron transfer from tyrosine 125 to oxidized methionine 127 of α-synuclein, but we also observed the reversed intramolecular electron transfer from methionine 127 to oxidized tyrosine 125. This statement is supported by evidence gathered from three different experiments. We performed comparative experiments with pulse radiolysis and linear sweep voltammetry between the synthetic peptide Ac-AYEMPSE-NH₂ and the synthetic peptide Ac-AYEAPSE-NH₂, which lacks methionine. The synthetic peptide Ac-AYEMPSE-NH₂ corresponds to residues 124 to 130 in the sequence of the C-terminal domain of α-synuclein.

We observed from the reaction of Ac-AYEMPSE-NH₂ with the hydroxyl radical that the formation and decay rates of the tyrosyl radical both increased in comparison to those of the reaction of Ac-AYEAPSE-NH₂ with the hydroxyl radical. This result can only be interpreted as a reversible intramolecular electron transfer between the oxidized methionine and the tyrosine.

We also measured the effective potentials of the two synthetic peptides by linear sweep voltammetry across a range of pH from 0.8 to 7.2. The slope of the $E$ vs. pH diagram of Ac-AYEMPSE-NH₂ is 49 mV/pH, instead of 59 mV/pH as expected in the case of a 1 e⁻ / 1 H⁺ proton–coupled electron transfer mechanism. In comparison, the slope of the $E$ vs. pH diagram of Ac-AYEAPSE-NH₂ is 59 mV/pH. This means that Ac-AYEMPSE-NH₂ can deliver more than one electron at the electrode surface. In all likelihood, a portion of all methionine can reduce the tyrosyl radical formed at the electrode surface, and tyrosine can be subsequently oxidized a second time at the electrode surface. We calculated from our measurements that the electrode potential of methionine in the vicinity of oxidized tyrosine is $E^\circ' = 1.14$ V.

Finally, we also observed from our experiments with monohydrogen ascorbate that the yield of ascorbyl radicals after the reduction reaction of the tyrosyl radical of Ac-AYEMPSE-NH₂ is
lower than expected. We interpret this observation as the result of various recombination reactions between the ascorbyl radical and the carbon centered (α-alkylthio) alkyl radical that result from the dissociation of the sulfur radical cation of methionine into the carbon centered radical and a proton.

This second result has implications for the chemistry of free radicals with α-synuclein. The resulting oxidative damage may not be exclusively located on the tyrosine residues of α-synuclein, but also on Met127. Once the sulfur radical cation of methionine deprotonates to (α-alkylthio) alkyl radicals, this electron transfer cannot be reversed, which in turn can lead to various possible products such as homocysteine. However, the only modification observed to date on methionine in α-synuclein exposed to oxidative conditions is sulfoxide.
RESUME

La maladie de Parkinson est une maladie en lien étroit avec le stress oxydatif qui résulte de lésions mitochondriales. Les neurones ont la plus haute concentration d’ascorbate monohydrogéné en comparaison d’autres tissus du corps. α-Synucléine est une protéine clé de l’étiologie de la maladie de Parkinson et est un composé majeur des corps d’inclusion appelés corps de Lewy. La protéine α-synucléine est susceptible au stress oxydatif et forme des dimères nitratés stables quand elle est exposée à des conditions de stress oxydatif. Ces dimères sont des protéines réticulées de manière covalent par des liens de o,o’-dityrosine. Il est admis que l’extrémité C-terminale de l’α-synucléine, et plus particulièrement le résidu Tyr125, favorise la formation de fibrilles.

Notre objectif était double. Notre premier but était d’examiner le comportement de l’extrémité C-terminale une fois soumise à des conditions de stress oxydatif. Notre approche a été de réaliser des expériences sur des peptides synthétiques qui correspondent aux derniers résidus 124 à 140 de la séquence de l’extrémité C-terminale de l’α-synucléine. Notre deuxième but était d’examiner si le réducteur ascorbate monohydrogéné peut réduire le radical tyrosyle formé par réaction avec le triazote(2N-N)(•) de manière suffisamment rapide pour éviter que le radical tyrosine de l’α-synucléine ne dimérisse.

Nous avons utilisé la radiolyse pulsée afin de produire le radical hydridoxygène(•) pour réaliser des conditions de stress oxydatif. Les expériences avec l’ascorbate monohydrogéné ont été réalisées avec des anions d’azide afin de convertir l’hydridoxygène(•) en triazote(2N-N)(•). Etant donné l’absence de tryptophane dans les peptides que nous avons utilisés pour nos expériences ou la protéine α-synucléine, le triazote(2N-N)(•) oxyde la tyrosine de manière ciblée.

Nous avons établi que l’ascorbate monohydrogéné réduit le radical tyrosyle de l’α-synucléine avant que celle-ci ne dimérisse. La constante de vitesse de la réduction du radicale tyrosyle (Tyr/O’) par l’ascorbate monohydrogéné (Hasc−) est de $k(Tyr/O’ + Hasc−) = 1.9 \pm 1.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. La constante de vitesse de la dimérisation de l’α-synucléine est de $k(\text{dimérisation}) = 1.7 \pm 0.8 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$. Si l’on prend comme base de calcul la plus basse concentration d’ascorbate monohydrogéné dans les neurones, à savoir [Hasc−] = 2.0 mM, la demi-vie du radical tyrosine est de 25 µs. Pour obtenir une demi-vie de 25 µs pour la dimérisation de l’α-synucléine, il
faudrait une concentration initiale de radical tyrosyle de 11 M. Dans ce cas de figure, le rapport entre les radicaux tyrosyle qui sont réduits et ceux qui dimérisent est de 50 :50. Pour obtenir un rapport de 99 :1, il faudrait encore une concentration de radical tyrosyle de 200 mM. Un concentration aussi haute de radical tyrosyle de l’α-synucléine est improbable, même en cas de conditions de stress oxydatif, et nous pouvons par conséquent affirmer que la réduction du radical tyrosyle de l’α-synucléine par 2 mM d’ascorbate monohydrogéné exclut la possibilité la dimérisation du radical tyrosyle de l’α-synucléine.

Notre deuxième résultat est aussi d’importance. De précédentes études ont rapporté que la méthionine oxydée peut oxyder une tyrosine située suffisamment près et contribuer ainsi à la formation du radical tyrosyle par un transfert d’électron intramoléculaire, supposément par effet tunnel. Nous avons non seulement observé un tel transfert d’électron intramoléculaire de la tyrosine 125 à la méthionine 127 oxydée de l’α-synucléine, mais nous avons aussi observé que ce transfert d’électron intramoléculaire est réversible, c’est-à-dire de la méthionine 127 à la tyrosine 125 oxydée. Cette affirmation est étayée par nos résultats obtenus par trois expériences différentes. Nous avons réalisé nos expériences avec le peptide synthétique Ac-AYEMPSE-NH₂, qui correspond aux résidus 124 à 130 de la séquence de l’extrémité C-terminale de l’α-synucléine. Nous avons réalisé des expériences comparatives entre le peptide synthétique Ac-AYEMPSE-NH₂ and le peptide synthétique Ac-AYEAPSE-NH₂, qui ne contient pas de méthionine.

Nous avons observé que la réaction de Ac-AYEMPSE-NH₂ avec le radical hydridooxygène(•) résulte en une formation et une décomposition du radical tyrosyle plus rapides que celles de Ac-AYEAPSE-NH₂. Ce résultat ne peut être interprété que comme un transfert d’électron réversible entre la méthionine oxydée et la tyrosine.

Nous avons aussi mesuré les potentiels effectifs des deux peptides synthétiques par voltammetrie à balayage linéaire à des valeurs de pH allant de 0.8 à 7.2. La pente du diagramme $E$ vs. pH de Ac-AYEMPSE-NH₂ est de 49 mV/pH au lieu de 59 mV/pH comme prévu dans le cas de figure d’un mécanisme de transfert d’électron couplé à un proton $1 e^- / 1 H^+$. En comparaison, la pente du diagramme $E$ vs. pH de Ac-AYEAPSE-NH₂ est de 59 mV/pH. Ce résultat veut dire que Ac-AYEMSPE-NH₂ échange plus d’un électron à la surface de l’électrode. En toute probabilité, une fraction de toutes les méthionines présentes en solution peut réduire le radical tyrosyle formé à la surface de l’électrode, la tyrosine peut subséquemment être oxydée une seconde fois à la
surface de l’électrode. Sur la base de nos mesures, nous avons calculé que le potentiel d’électrode de la méthionine proche d’une tyrosine oxydée est de 1.14 V.

Enfin, nous avons aussi observé de nos résultats avec l’ascorbate monohydrogéné que le rendement du radical ascorbyle après la réduction du radical tyrosyle de Ac-AYEMSP-E-NH₂ par l’ascorbate monohydrogéné est plus bas que prévu. Nous interprétons cette observation comme le résultat de différentes réactions de recombinaison entre le radical ascorbyle et le radical carbone centré (α-alkylthio) alkyl qui résulte de la dissociation du radical cation sulfure de la méthionine en un radical carbone centré et un proton.

Ce deuxième résultat a des implications pour la chimie des radicaux libres avec l’α-synucléine. Les dégâts résultants de stress oxydatif ne sont pas exclusivement situés sur les résidus de tyrosine, mais aussi sur Met127 de l’α-synucléine. La dissociation du radical cation de sulfure de la méthionine en un radical (α-alkylthio) alkyle n’est pas réversible, ce qui peut résulter en divers produits possibles comme une homocystéine en place de la méthionine. Cependant, la seule modification de la méthionine de l’α-synucléine soumise à des conditions de stress oxydatif rapportée à ce jour dans la littérature reste le sulfoxyde.
Chapter 1 INTRODUCTION

This project investigates the chemistry of the protein \( \alpha \)-synuclein, a main component of toxic inclusion bodies found in neurons, under oxidative conditions. These inclusion bodies are known as Lewy bodies and are linked to the etiology of Parkinson’s disease. The dominant theory about the cause of Parkinson’s disease is that oxidative stress causes cell damage. In the following introduction, we will discuss the etiology of Parkinson’s disease, the genetic and molecular causes behind the generation of partially reduced oxygen species, and the link between oxidative stress and the aggregation of \( \alpha \)-synuclein into fibrils. We also want to investigate whether the antioxidant ascorbate can prevent the formation of \( \alpha \)-synuclein oligomers and thus review the essential knowledge about monohydrogen ascorbate in the brain.

\( \alpha \)-Synuclein quickly drew the attention of the research community due to its strong link to the etiology of various neurodegenerative diseases. It is a protein described as natively unfolded because its lack of structure in solution and it aggregates spontaneously, albeit slowly. This propensity to form fibrils was initially linked to point mutations in the sequence of \( \alpha \)-synuclein, but as research developed, oxidative stress was shown to play a role as well. The formation of \( \alpha \)-synuclein fibrils starts after a long lag-phase during which \( \alpha \)-synuclein aggregates into oligomers. The formation of dityrosine bonds stabilizes these oligomers. This fact drew our attention because it suggests that tyrosine radicals are formed in the first place. Therefore, we decided to investigate the chemical behavior of \( \alpha \)-synuclein under conditions of oxidative stress.

The proximity of Met127 to Tyr125 theoretically allows electron transfer between the two residues to occur. We believe that this proximity influences the chemical behavior of \( \alpha \)-synuclein under oxidative conditions. We review the theoretical basis of electron tunneling through a protein and the experimental evidence for the intramolecular electron transfer from tyrosine to oxidized methionine at the end of this chapter.

1.1 Parkinson’s disease
1.1.1 Overview

Parkinson’s disease is the most frequent neurodegenerative movement disorder with a worldwide estimate of 4 million affected patients, mostly elderly people. This estimate totals about 5% of people above 60 years old. In Switzerland, there are 15’000 people with a diagnosis of Parkinson’s disease. The estimated average annual treatment cost is $54’000 per patient. It is an expensive disease to treat and, to date, there is no cure available.

Parkinson and Charcot described the clinical symptoms of Parkinson’s disease in the 19th century. The four cardinal features of Parkinson’s disease include tremor at rest, rigidity, akinesia, and postural instability. The typical pathological hallmark of early presymptomatic events in Parkinson’s disease development is intraneuronal proteinaceous inclusion bodies in the substantia nigra pars compacta and in other regions of the central and peripheral nervous system. These intraneuronal inclusion bodies develop through several stages of Parkinson’s disease, either as spindle-like Lewy neurites or as granular aggregations called Lewy bodies. Lewy neurites and Lewy bodies develop in the somata of neurons vulnerable to these aggregation processes. Lewy neurites and Lewy bodies consist mainly of aggregates of fibrillar α-synuclein proteins and ubiquitin.Remarkably, the development of Parkinson’s disease follows patterns, and the evolution of the distribution of lesions is predictable. The lesions in Parkinson’s disease develop first in the allocortex, then in the substantia nigra pars compacta, and finally affect the neocortex. Consequently, Parkinson’s disease symptoms appear chronologically from non-motor symptoms characterized by olfactory deficits, sleep disorders and constipation (Figure 1, Stages 1-3) to the motor symptoms described above. Finally, the late stage of Parkinson’s disease settles in with accompanying cognitive decline and dementia (Figure 1, Stages 4-6). Vulnerable neurons are projection neurons with long, thin, and poorly myelinated axons. 95% of Parkinson’s disease cases are sporadic, and 5% of Parkinson’s disease cases have a genetic cause.

Oxidative stress is strongly linked to the etiology of Parkinson’s disease. Oxidative stress is defined as the “disequilibrium between the generation of ROS and their detoxification by endogenous antioxidants leading to macromolecular damage.” As detailed below, both environmental and genetic factors have been identified as causes of mitochondrial dysfunction and subsequent uncontrolled production of PROS in neurons.
1.1.2 Environmental factors and Parkinson’s disease

To date, there is no convincing data to substantiate that a specific toxin induces Parkinson’s disease. The three toxins that lead to symptoms that also found in Parkinson’s
disease are 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), (2R,6aS,12aS)-1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9-dimethoxychromeno[3,4-b]furo(2,3-h)chromen-6-one (rotenone), and 1,1'-dimethyl-4,4'-bipyridinium dichloride (paraquat). There is, however, no mechanism to explain how these effects lead to Parkinson’s disease symptoms.\textsuperscript{26,27}

1.1.2.1 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

MPTP is a side-product of the chemical synthesis of an illegal drug, 1-methyl-4-phenyl-4-propionoxy-piperidine (MPPP). In 1982, drug users with a history of MPPP abuse developed a syndrome nearly identical to that of Parkinson’s disease. The consumption of MPTP was linked to the development of this symptom.\textsuperscript{28}

MPTP converts into 1-methyl-4-phenylpyridinium ion (MPP\textsuperscript{+}) (Figure 2) through the enzymatic action of the monoamine oxidase B. MPP\textsuperscript{+} then enters dopaminergic neurons via dopamine transporters where it inhibits mitochondrial complex I\textsuperscript{29}. The result is damage to the nigrostriatal dopaminergic pathway\textsuperscript{30}. MPTP is used to obtain animal models for Parkinson’s disease by intravenous or intraperitoneal injection\textsuperscript{31}, yet the validity of the model is controversial\textsuperscript{31-37}. The fact that MPTP inhibits mitochondrial complex I lends weight to the hypothesis of the mitochondrial dysfunction as a pathway for neurodegeneration\textsuperscript{38,39}.  

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1.12.2 1,1’-Dimethyl-4,4’-bipyridinium dichloride (Paraquat)

The herbicide 1,1'-dimethyl-4,4'-bipyridinium dichloride (paraquat) has a similar chemical structure to MPTP (Figure 2). Paraquat poorly crosses the blood-brain barrier but irreversibly damages the dopaminergic neurons upon direct exposure\(^{30}\). Its distribution in the central nervous system does not correspond to any known enzymatic or neuroanatomic distribution\(^{40}\). Day et al. provided evidence that paraquat uses oxonitrogen(\(\cdot\)) synthase to produce dioxide(\(\cdot\)\(^{1–}\))\(^{41}\). Systemic injection of paraquat is a method of choice to reproduce the major aspects of Parkinson’s disease in animal models\(^{42-44}\).
1.1.2.3 \((2R,6aS,12aS)-1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9-dimethoxychromeno[3,4-b]furo(2,3-h)chromen-6-one\) (Rotenone)

Rotenone is a lipophilic compound (Figure 2) and infiltrates all organs\(^45\). Rotenone was initially identified as a Parkinson’s disease symptom-inducing compound that targets nigrostriatal dopaminergic neurons\(^46\), but later studies put these results into question. Rotenone was shown to affect other types of neurons without specific target\(^47\) and no specific take-up by dopaminergic neurons could be demonstrated\(^48\). Like 1-methyl-4-phenylpyridinium ion and 1,1’-dimethyl-4,4’-bipyridinium dichloride, rotenone inhibits mitochondrial complex I\(^49\).

1.1.2.4 5-(2-Aminoethyl)benzene-1,2,4-triol (6-Hydroxydopamine)

5-(2-Aminoethyl)benzene-1,2,4-triol (6-hydroxydopamine) (Figure 3) cannot cross the blood-brain barrier. As a result, it has to be directly injected stereotaxically into the substantia nigra pars compacta or the striatum\(^50,51\). 6-Hydroxydopamine accumulates in the cytosol where it inhibits complex I. This inhibition induces the generation of PROS and of quinones. These pro-oxidants attack nucleophillic groups of biological macromolecules, thereby inactivating them\(^37,52,53\). 6-Hydroxydopamine induces brain damage that differs from those observed in Parkinson’s disease and does not lead to the formation of Lewy bodies. Its toxicity is relatively selective for monoaminergic neurons as a result of preferential uptake by dopamine and noradrenergic transporters\(^54\). 6-Hydroxydopamine damages 80 % of dopaminergic neurons within 24 hours\(^51,53,55,56\) and induces development of motor impairment\(^37,57\). In fact, 6-hydroxydopamine injections provoke too drastic and immediate neuronal damage to be considered as a valid model for Parkinson’s disease\(^53\).
1,1’-dimethyl-4,4’-bipyridinium dichloride is reduced by complex I and forms the 1,1’-dimethyl-4,4’-pyridinium pyridin-1-yl radical, which in turn is oxidized by dioxygen. This results in the formation of dioxide($\bullet$1–). Dioxide($\bullet$1–) reacts at rate constants $k \leq 10^6 \text{ M}^{-1}\text{s}^{-1}$ with amino acids, and itself behaves like a mild reductant, and its hydrogenated form behaves like an oxidant. Dioxide($\bullet$1–) reacts with nitrogen monoxide at a rate constant of $k = 1.6 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$ to form peroxynitrite. Peroxynitrous acid has high one- and two-electrode potentials at pH 7 of $E^\circ'(\text{ONOOH},\text{H}^+/\text{NO}_2^-,\text{H}_2\text{O}) = +1.6\pm0.1 \text{ V vs. NHE}$ and $E^\circ'(\text{ONOOH},\text{H}^+/\text{NO}_2^-,\text{H}_2\text{O}) = +1.3\pm0.1 \text{ V vs. NHE}$, respectively. Detection of nitrated tyrosine 108 in Cu,Zn-superoxide dismutase provided evidence that peroxynitrie forms in activated macrophages. Peroxynitrous acid oxidizes lipids and thiols. Finally, peroxynitrous acid also nitrates and hydroxylates tyrosine. Cu,Zn-superoxide dismutase catalyzes the dismutation of dioxide($\bullet$1–) into oxygen and...
hydrogen peroxide. Cu,Zn-superoxide dismutase has a rate constant with dioxide(•1–) of \[ k = 2.0 \times 10^9 \text{ M}^{-1}\text{s}^{-1} \] Under the assumption that the concentration of Cu,Zn-superoxide dismutase is 10 µM and that of nitrogen monoxide is 10 nM, we find from the rate constants mentioned above the pseudo-first-order rate constants of \[ k_{\text{obs}} = 2.0 \times 10^4 \text{ s}^{-1} \] and \[ k_{\text{obs}} = 1.6 \times 10^2 \text{ s}^{-1} \] respectively. Under these conditions, only 1 % of dioxide(•1–) reacts with nitrogen monoxide. Under oxidative stress conditions though, when enough dioxide(•1–) and nitrogen monoxide are produced, it is conceivable that the formation of peroxynitrite explains the damage to the cell.

1.1.3 Genetics of Parkinson’s disease

Not only are certain toxins putative etiological factors in Parkinson’s disease related to mitochondrial dysfunction and oxidative stress, but some genes are also known as etiological factors of Parkinson’s disease. One has to bear in mind that Parkinson’s disease is a multifactorial disease and research on genetic causes of Parkinson’s disease is still on-going. Thorough reviews of loci associated with Parkinson’s disease list many of these genes. We discuss in this section four genes whose effects best illustrate the involvement of genetic factors in disruption of cellular energetics pathways: PTEN-induced putative kinase 1 (PINK1), parkin, ARF, and DJ-1.

1.1.3.1 PINK1 and Parkin genes and their involvement in the biology of the mitochondrion

PINK1 and parkin have a mitochondrial quality-control role. PINK1 belongs to the family of protein kinases and coats the mitochondrial outer membrane with its kinase domain facing the cytosol. Parkin belongs to the E3 ubiquitin ligase family and localizes in the cytoplasm. It can mono- and polyubiquitinate a large array of proteins to target proteins for degradation.

PINK1 and parkin are both associated with familial Parkinson’s disease upon mutations of key residues. Oxidative stress inactivates parkin in sporadic Parkinson’s disease. The nonreceptor tyrosine kinase, c-Abl, phosphorylates tyrosine 143 of parkin, which as a result then loses its
activity in cases of sporadic Parkinson’s disease\textsuperscript{78}. Early onset of Parkinson’s disease, often before the appearance of Lewy bodies, results from mutations on parkin. Parkin mutations cause a large proportion of familial cases of Parkinson’s disease\textsuperscript{76}. Parkinson’s disease linked mutations impair parkin activity and its interactions with E2 enzymes such as UbcH7 or UbcH8\textsuperscript{75,79}. The accumulation of parkin substrates upon inactivation of parkin may contribute to the degeneration of dopaminergic neurons\textsuperscript{25}.

Parkin regulates levels of parkin interacting substrate (PARIS), in that inactivation of parkin brings about the accumulation of PARIS in the brain of patients with Parkinson’s disease. This accumulation subsequently leads to the downregulation by transcriptional repression of the expression of the peroxisome proliferator activated receptor gamma (PPAR-\(\gamma\)) coactivator-1\(\alpha\) (PGC-1\(\alpha\)) gene. The inhibition of PGC-1\(\alpha\) in turn stalls the reproduction of mitochondria, which leads to the progressive loss of dopaminergic neurons. This pathway is known as the parkin-PARIS-PGC-1\(\alpha\) pathway (Figure 4)\textsuperscript{80}.

Other parkin substrates such as aminoacyl-tRNA-synthesase-interacting multifunctional protein type 2 (AIMP2)\textsuperscript{81,82}, and far upstream element-binding protein 1 (FBP-1)\textsuperscript{83} may also play a role in neurodegeneration.

![Figure 4: Parkin inhibits PGC1-\(\alpha\) expression through PARIS. Adapted from Martin et al. \textsuperscript{25} with permission from the rights owner.](image-url)
1.1.3.2 PINK1 and parkin interactions in mitochondrial disruption

Models of PINK1 knock-out and parkin knock-out *Drosophila melanogaster* display similar phenotypes, which consist of locomotor impairment, apoptotic muscle degeneration, reduced lifespan, mitochondria with tumescent and thin appearance, and loss of dopaminergic neurons\(^{84,85}\). Flies with PINK1 or parkin deficits have reduced resistance to exogenous oxidative stressors. Antioxidant overexpression can however prevent neuron lysis\(^{86,87}\). Many studies indicate that PINK1 and parkin make a pathway in which PINK1 is upstream\(^{77,88-90}\). Upon the loss of membrane potential, mitochondria are depolarized and identified for autophagy in a PINK1-dependent manner through the recruitment of parkin\(^{91,92}\). After its recruitment at the mitochondrial surface, parkin ubiquitinates its targets\(^{93-98}\), which tags damaged mitochondria and segregates them for degradation in a P62-mediated process\(^{99}\). Pathogenic mutations in PINK1 or parkin seem to disrupt parkin recruitment, substrate ubiquitination, and mitophagy (Figure 5)\(^{99,100}\).
1.1.3.3 smARF depolarizes mitochondrial membrane

Short mitochondrial ARF (smARF) is a short isoform of p19ARF, a tumor suppressor. smARF depolarizes the mitochondrial membrane by localizing on them in neurons, which consequently triggers mitophagy\textsuperscript{101}. PINK1 and parkin are essential for smARF to promote mitophagy. Parkin and PINK1 are downstream factors in the autophagy pathway\textsuperscript{102}.

Figure 5: Diagram that shows the genetic and sporadic causes of Parkinson’s disease and their potential interactions and effects. Both genetic and sporadic causes result in mitochondrial dysfunction. DJ-1, PINK1, and Parkin may directly cause mitochondrial dysfunction. Note the upstream positions of Parkin and PARIS in the regulatory cascade of mitochondrial biogenesis (Parkin → PARIS → PGC–1α → NRF–1). Dominant mutations in POLG, LRRK2, and α-synuclein cause parkinsonism. Adapted from Martin et al. \textsuperscript{25} with permission from the rights owner.
1.1.3.4 DJ-1 (PARK7) interacts with the mitochondrial membrane to prevent neuronal losses

The various functions of DJ-1 include transcriptional regulation, antioxidative stress reactions, and chaperone protease\textsuperscript{103}. Homozygous loss of function mutations in DJ-1 lead to the early onset of Parkinson’s disease\textsuperscript{104}. DJ-1 is neuroprotective under oxidative stress\textsuperscript{105-109}. In human dopaminergic neuroblastoma cells, DJ-1 knock-down leads to mitochondrial depolarization and fragmentation (Figure 5). As a result, DJ-1 might be important for the maintenance of the mitochondrial pool. Additionally, antioxidant treatment prevents mitochondrial depolarization and oxidative stress results in DJ-1 deficiency\textsuperscript{110-112}. DJ-1 has peroxiredoxin-like peroxidase activity and its protective mechanism rests partly on its H$_2$O$_2$ scavenging ability\textsuperscript{107}. Its most potent action is due to its ability to bind RNA upon oxidative stress and regulate the signaling pathway of oxidative stress-dependent kinases\textsuperscript{113,114}. As a result, DJ-1 upregulates synthesis of glutathione\textsuperscript{115}.

1.2 $\alpha$-Synuclein

1.2.1 Overview

Maroteaux \textit{et al.} identified in 1988 the first gene of the synuclein protein family, which was found exclusively in the nervous system of the pacific electric ray \textit{Torpedo californica}\textsuperscript{116}. Shortly afterwards, $\alpha$-synuclein was linked to the formation of fibrils and Lewy bodies\textsuperscript{14,117,118}. The importance of $\alpha$-synuclein and Lewy bodies lays beyond the domain of the etiolo of Parkinson’s disease, because many studies reported the detection of $\alpha$-synuclein positive inclusions in brains of patients with neurodegenerative diseases, which, apart from Parkinson’s disease, include Alzheimer’s disease, multiple system atrophy, and dementia with Lewy bodies\textsuperscript{119-126}.

The first factor identified as the cause of the formation of fibrils was a variety of mutations in $\alpha$-synuclein. The mutations Ala30Pro, Glu46Lys, His50Gln, and Ala53Thr, were shown to be
responsible for the autosomal dominant, inheritable form of Parkinson’s disease\textsuperscript{123,127-129}. The duplication and the triplication of the $\alpha$-synuclein gene also leads to an autosomal dominant inheritable form of Parkinson’s disease\textsuperscript{130,131}.

1.2.2 The $\alpha$-synuclein gene and its transcription

The human $\alpha$-synuclein gene is referred to as SNCA. It is located on chromosome 4q21.3-q22 and covers a region of 111 kb\textsuperscript{132-134}. The gene consists of seven exons, out of which five are coding\textsuperscript{135}. The gene has four isoforms, called SNCA96, SNCA112, SNCA126, and the full-length isoform SNCA140\textsuperscript{136}.

1.2.3 Properties of the $\alpha$-synuclein protein

The $\alpha$-synuclein protein has a molecular mass of 14 460 Da and three sequence domains: the amphipathic N-terminal domain, the hydrophobic NAC region, and the acidic C-terminal domain\textsuperscript{137}. The sequence of residues 1-60 makes up the N-terminal domain, and contains six imperfect repeats of the consensus motif KTKEGV. Upon binding to membranes and synthetic lipid vesicles, the N-terminal domain forms amphipathic $\alpha$-helices (Figure 7)\textsuperscript{138-140}. The highly hydrophobic NAC domain extends from residues 60 to 95 and plays a central role in fibril formation (Figure 7)\textsuperscript{141}. The acidic C-terminal domain spans residues 96 to 140 and remains in a disordered state, even upon binding of $\alpha$-synuclein to a lipid membrane (Figure 7)\textsuperscript{142}. Although the $\alpha$-synuclein does not exist in invertebrates, it is exceptionally conserved in the vertebrate subphylum. The sequence comparison between those of mammals and birds shows that most amino acid differences are found in the C-terminal domain, with some amino acid differences in the N-terminal and NAC domains. Intriguingly, the Parkinson’s disease linked mutation Ala53Thr occurs in most vertebrate sequences of $\alpha$-synuclein, except in those of primates and humans\textsuperscript{137}.
1.2.4 The structure of α-synuclein

1.2.4.1 Disordered structure in solution

α-Synuclein is described as a natively unfolded protein due to the absence of a stable secondary structure in solution\textsuperscript{144,145}. Heat does not induce the precipitation of α-synuclein. The accepted estimate of the Stokes radius of α-synuclein is 34 Å, which corresponds to a typical 58 kD globular protein. This molecular weight has to be compared to that of α-synuclein, 14 kD. During an SDS-PAGE run, the band that corresponds to α-synuclein migrates slowly with an apparent weight of 19 kD\textsuperscript{144}. 

Figure 6: Top; Helical wheel projection of α–synuclein. Black circles; apolar residues. White circles; polar and charged residues. Charged residues are indicated with + and – to signify their charge. Bottom; α-Synuclein sequence with the 6 imperfect repeats of KTKEGV highlighted in red. Reproduced from George \textit{et al.}\textsuperscript{143} with permission from the rights owner.
1.2.4.2 Partial conformation formation upon binding to membranes

A theoretical wheel projection of α-synuclein shows a clear segregation of polar and non-polar residues on opposite faces of the α-helix, which is the feature of an amphipathic helix (Figure 6)\textsuperscript{143}. This theoretical projection wheel proves accurate because α-synuclein binds to anionic phospholipid vesicles and synthetic lipid-membrane, such as SDS micelles\textsuperscript{146}. Circular dichroism evidence showed that α-synuclein adopts an α-helix as a secondary structure upon binding to membranes (Figure 7)\textsuperscript{147}. Mutation experiments, where the Ala30Pro mutant exhibits reduced membrane-binding affinity \textit{in vitro} and in yeast, demonstrated that the N-terminal domain binds to phospholipids\textsuperscript{148,149}. An \textit{in vivo} study confirmed that the mutation Ala30Pro leads to the loss of affinity by the N-terminal domain to the lipid membrane\textsuperscript{150}. The α-synuclein region that interacts with lipid membranes spans the residues 1-102. The C-terminal does not contribute to the binding and remains unstructured\textsuperscript{139,146}. NMR based structural studies reported that interaction between vesicles of lipid membranes and α-synuclein occurs through the formation of two antiparallel broken α-helices. The first helix spans over residues 3-37 and the second helix spans over residues 45-92\textsuperscript{138,151-153}. Interestingly, the curvature of the vesicles either constrains α-synuclein into an extended single α-helix or two broken α-helices\textsuperscript{154}.
Figure 7: Annotated structure of α–synuclein. Middle; Full NMR structure of α-synuclein bound to SDS-micelles in solution. PDB structure: 1XQ8. Orange; N-terminal domain. Blue; NAC domain. Red; C-terminal domain. Most of the N-terminal and NAC domains form two α-helices when α-synuclein binds to highly curved micelles. Residues in stick representation: Grey; Locations of the four mutations Ala30Pro, Glu46Lys, His50Gln, and Ala53Thr. Green; Locations of the phosphorylated residues Ser87, Tyr125, Tyr133, and Tyr136. Blue; Location of Met127. Yellow; Location of metal cations-binding residues.

1.2.5 Functions of α-synuclein

1.2.5.1 Regulation of vesicle trafficking

Studies that investigated the role of α-synuclein in neurons quickly ruled out the involvement of α-synuclein in synaptogenesis\textsuperscript{155,156}, but pointed out its role in the regulation of vesicle trafficking. Various studies on the down-regulation of α-synuclein showed that α-synuclein might be involved in the process of vesicle trafficking from reserve pools to release sites\textsuperscript{157-159}. α-Synuclein may also regulate exocytosis by inhibiting the vesicle priming step\textsuperscript{160}. 
1.2.5.2 Regulation of phospholipase D activity

In vitro and in vivo studies report that α-synuclein binds preferentially to acidic phospholipids\textsuperscript{147,148,150}. Some studies showed that both wild-type and mutant α-synuclein species inhibit phospholipase D, with their inhibitory potency increased by phosphorylation of Tyr125. Phospholipase D cleaves phosphatidylcholine into the signal molecule phosphatidic acid and choline, the precursor to the neurotransmitter acetylcholine\textsuperscript{161,162}. The regulation of various signals at the plasma membrane and synaptic maintenance depend on the regulation of phospholipase D, as normal lipid balance will ensure cell growth and the release of neurotransmitters\textsuperscript{162}. The loss of α-synuclein monomers to oligomers and fibrils may disrupt the composition of membrane lipids and induce cell toxicity.

1.2.5.3 Role as a chaperone

α-Synuclein prevents precipitation of various proteins even from heat-induced denaturation\textsuperscript{163-165}. The C-terminal domain of α-synuclein provides chaperone activity, because it confers its thermal stability to α-synuclein\textsuperscript{166}. However, the N-terminal and NAC domains of α-synuclein display no chaperone activity\textsuperscript{163,166}.

1.2.5.4 Debated neuroprotective role

In addition to the cytotoxic action of α-synuclein, the role of α-synuclein as a neuroprotective agent is still debated. There are contradicting reports on whether or not α-synuclein confers resistance to H\textsubscript{2}O\textsubscript{2} and MPP\textsuperscript{+} induced oxidative stress\textsuperscript{167-170}. Two different studies show a correlation between the expressions of α-synuclein and resistance to 6-hydroxydopamine and paraquat induced oxidative stress\textsuperscript{171,172}. Beside its potential role as a neuroprotective agent, the expression of α-synuclein also plays a rescue role in apoptotic cells\textsuperscript{173}. 
1.2.6 α-Synuclein role in neurotoxicity

1.2.6.1 Overview

Neurodegeneration takes place chiefly in the substantia nigra pars compacta, precisely in the dopaminergic neurons domain. Since expression of the α-synuclein extends to the whole brain, how can α-synuclein specifically induce damages in dopaminergic neurons? However, the molecular basis of cell toxicity in dopaminergic neurons remains vague. A possible link between α-synuclein and neuronal death may be that α-synuclein regulates the production, the storage, the release, and the reuptake of dopamine (Figure 3).

1.2.6.2 α-Synuclein and regulation of the levels of dopamine in the neuronal cytoplasm

α-Synuclein negatively modulates the gene expression of the tyrosine hydroxylase and modulates the activity of the enzyme by allosteric inhibition. As a result, the amount of cytoplasmic dopamine decreases when α-synuclein is expressed. The vesicular monoamine transporter 2 (VMAT2) regulates the vesicular storage of dopamine. The overexpression of Ala53Thr α-synuclein leads to a decrease in the levels of VMAT2, which results in an increase of cytoplasmic dopamine. The mutants Ala53Thr and Glu46Lys, but not Ala30Pro, prevent the exocytosis of the synaptic vesicles. There is a molecular transporter, called a dopamine transporter, which is dedicated to the uptake of dopamine into neurons. α-Synuclein binds to the dopamine transporter, increases the number of dopamine transporters at the neuronal membrane and as a result promotes the uptake of dopamine. Parkin competes against the binding of α-synuclein and prevents dopamine-induced cytotoxicity.

To sum up, if the availability of α-synuclein is disrupted, this will affect the homeostasis of cytoplasmic dopamine necessary for the preservation of dopaminergic neurons. Interestingly, α-synuclein and dopamine synergistically harm the dopaminergic neurons. Oxidized dopamine potently inhibits the fibril formation of α-synuclein and promotes the formation of potentially cytotoxic oligomers of α-synuclein.
1.2.6.3 Mitochondrial dysfunction

α-Synuclein binds directly to the outer membrane of the mitochondrion on the cytosol side and impairs mitochondrial structure and function as a result. This happens in the human brain\textsuperscript{184} and in cell models in culture\textsuperscript{185-187}. α-Synuclein binds purified mitochondria \textit{in vitro}\textsuperscript{185} and, as Parkinson’s disease progresses, more α-synuclein binds to mitochondria, possibly due to the acidification of the cytosol\textsuperscript{185}. Uptake of α-synuclein by the mitochondrion causes fission of the organelles, a depletion of intracellular ATP, and an increase in PROS, which eventually leads to cell death\textsuperscript{184,186}.

1.2.6.4 Are Lewy bodies neuroprotective or neurotoxic?

Neurotoxicity is strongly linked with Lewy bodies. The observation that mutations linked to Parkinson’s disease foster the fibril formation of α-synuclein in vitro and in animal models of synucleopathies validate this link\textsuperscript{188-191}. Several findings, however, suggest a broader role for Lewy bodies. Brains of patients with Parkinson’s disease still contain surviving neurons with Lewy bodies\textsuperscript{14}. The extent of formation and neurodegeneration of Lewy bodies in humans does not correlate. Lewy bodies were found in brains of dead patients that had lived without signs of Parkinson’s disease or neurodegeneration\textsuperscript{192,193}. This apparent contradiction was resolved when it was shown that fibrils are not the reason for cytotoxicity, but that some specific oligomer species of α-synuclein are. The fibrils play a neuroprotective role when they trap the toxic oligomers. The following evidence backs up this hypothesis. Several mutations (Ala30Pro, Ala56Pro, Glu57Lys, and Glu35Lys) foster the formation and the stabilization of α-synuclein oligomers, which in turn aggravate neurotoxicity in vivo\textsuperscript{194-197}. α-Synuclein oligomers affect the structure of neuronal microtubules\textsuperscript{198}. Known inducing factors of stable oligomers include metals\textsuperscript{199}, polyunsaturated fatty acids\textsuperscript{200-202}, dopamin\textsuperscript{182,183}, lipids\textsuperscript{203}, and interfacial effects of the air-water interface\textsuperscript{204}. Some specific α-synuclein oligomers have been identified as neurotoxicity inducing\textsuperscript{199,202}. The molecular link to the formation of oligomers of α-synuclein, however, remains elusive to this date.
1.2.7 Fibril formation

The presence of α-synuclein in Lewy bodies was discovered shortly after the discovery of the α–synuclein gene\textsuperscript{125,126}. Immunogold labelling and staining with fibril binding fluorescent dyes like Thioflavin S and Congo Red experiments confirmed the fibrillar nature of these α-synuclein positive inclusions\textsuperscript{137}. Fibril formation occurs \textit{in vitro} by dissolving α-synuclein and these fibrils are similar to fibrils found \textit{post mortem} in the brain of patients with Parkinson’s disease\textsuperscript{205,206}. The molecular mechanisms that underlie α-synuclein fibril formation remain unexplained. Several studies hint at the NAC domain as the determinant factor for fibril formation (Figure 8)\textsuperscript{141,207-212}.

![Figure 8: Electron micrographs of α-synuclein fibrils. A; Recombinant wild-type human α-synuclein. B; Recombinant human A30P α-synuclein mutant. Reproduced from Serpell et al. 2000\textsuperscript{211} with permission from the rights owner.](image)

1.2.7.1 Overall fibril formation process

The formation of α-synuclein fibrils follows a well-defined pathway. It starts with an initial lag phase during which α-synuclein aggregates into oligomers. Then fibril formation and elongation set in with a rapid growth phase. If preformed shortened fibrils are added to a solution of α-synuclein monomers, the lag phase is eliminated. These two observations suggest a nucleation-dependent mechanism. The oligomers serve as competent nuclei for fibril elongation. Fibrils formation eventually reaches a plateau phase, where fibril and monomer concentrations are at an equilibrium\textsuperscript{213}. 
1.2.7.2 Characterization of oligomers

Although the observations described above outline the importance of oligomers in the formation of α-synuclein fibrils, no oligomer was directly observed in the brain. Various studies using western blots and oligomer-specific ELISA, however, revealed the presence of SDS resistant oligomers in brain homogenates and samples of cerebrospinal fluid from patients with Parkinson’s disease\textsuperscript{214,215}. Experiments on animal models of synucleinopathies coupled to oligomer-specific immunoblotting methods identified the formation of oligomers in endoplasmic reticulum\textsuperscript{216}. The formation of oligomers is favored by the phosphorylation of Ser129\textsuperscript{217}. Transmission electronic microscopy and atomic force microscopy studies reported that the initial conditions set for the formation of α-synuclein oligomer or fibril constrain the oligomer shapes into either spherical, pore-like, or amorphous structures\textsuperscript{199,218,219}. These oligomers quickly disappear and their size varies from 35 to 125 nm\textsuperscript{219}.

1.2.7.3 Fibrils characterization

An X-Ray fiber diffraction study revealed that α-synuclein fibrils present a cross-β-structure with a distance between β-strands of about 4.7-4.8 Å and a distance between β-sheets of about 10 Å\textsuperscript{211}. Transmission electronic microscopy and atomic force microscopy studies reported that the resulting fibrils are typically 10 nm wide and 0.1 - 10 µm long\textsuperscript{205,206}. One transmission electronic microscopy study linked the formation of oligomers to the formation of fibrils. Monomers first assemble into rod-shaped protofibrils with β-sheet structures. Upon extension of the protofibrils in a direction perpendicular to β-strands, they can then either retain a linear shape or take an annular shape. Linear protofibrils form fibrils through lateral interaction with each other. Annular protofibrils first open their ring into two linear protofibrils that assemble through lateral interaction (Figure 9)\textsuperscript{220}. 
1.2.7.4 Various factors of fibril formation

1.2.7.4.1 Genetic factors

Wild-type α-synuclein oligomerizes and forms fibrils spontaneously in solution; within 48 hours, the first fibrils are observed. All Parkinson’s disease linked mutations A30P, A53T, E46K, and H50Q enhance the oligomerization of α-synuclein in comparison to the wild-type protein. Each mutation has a different impact on formation rate, stabilization, shape, and size distribution of the fibrils.\textsuperscript{206,218,221-225}
1.2.7.4.2 Lipids

It is known that α-synuclein binds to synthetic phospholipids in vitro and to synaptic vesicles in vivo\textsuperscript{147,150}. α-Synuclein’s propensity to aggregate increases upon binding to lipid membranes, which suggests that interaction with neuronal membranes could cause α-synuclein fibril formation\textsuperscript{180,225,226}. Interaction between oxidized lipid vesicles and α-synuclein leads to the oxidation of Met1 and Met5, but not Met116 and Met127 of α-synuclein\textsuperscript{227}.

1.2.7.4.3 Post-translational modifications

α-Synuclein units found in Lewy bodies display various post-translational modifications, with the major one being the phosphorylation of Ser129\textsuperscript{228}. Phosphorylation at Ser87 or the mutation Ser87Glu inhibits the formation of fibrils\textsuperscript{229,230}. The kind of kinase that phosphorylates serine 129 influences the formation rate of fibrils\textsuperscript{228,231-233}. The phosphorylation of all three tyrosine residues Tyr125, Tyr133, and Tyr136 inhibits the formation of oligomers and inclusions of α-synuclein\textsuperscript{234}.

1.2.7.4.4 Metals

The C-terminal domain of α-synuclein is rich in negative charges at physiological pH. The residues 119-124 are the binding site for different metal ions\textsuperscript{235-237}. Various cations bind to α-synuclein and each binding cation specifically modifies the rate of fibril formation and the final fibril shape\textsuperscript{236}. Upon binding to the C-terminal domain, copper (II) enhances the rate of the fibril formation\textsuperscript{235,238}. Al(III) is more potent than Al(II) in fostering the formation of fibrils\textsuperscript{239}. Specific metal cations also induce specific oligomeric species of α-synuclein, such as the binding of copper (II) with α-synuclein, which was shown to induce the oligomeric unit cytotoxic\textsuperscript{199}. Iron(III) induces oligomers of α-synuclein that assemble into permeating pores across a lipid-bilayer\textsuperscript{240}. 
1.2.7.4.5 Oxidative stress

Treatment of neurons with rotenone leads to modifications (phosphorylation, nitration, amination, oxidation) on five specific residues of α-synuclein; Met116, Tyr125, Met127, Tyr133, and Tyr136\(^{241}\). In cell-based models, oxidative stress inducing conditions, such as treatment with peroxynitrite, rotenone, and FeCl\(_2\), increase the aggregation of α-synuclein and fibril formation\(^{168,242,243}\). The other major oxidative stress modification to α-synuclein is methionine oxidation, either to sulfoxide or to sulfone. Oxidized dopamine can interact directly with α-synuclein and induce the oligomerization of the latter\(^{244}\). The molecular effects of dopamine toxicity were also investigated. It was established that dopamine metabolism is associated with α-synuclein related cytotoxicity. Neurons were transformed with one of the four following mutants of α-synuclein, Tyr125Asp, Met127Ala, Met116Ala/Met127Ala, or Ser129Ala, with all of these mutants exhibiting resulting lower levels of methionine sulfoxide\(^{245}\). When dopamine is co-incubated with Y\(_{125EMPS129}\), the quantity of H\(_2\)O\(_2\) produced by autoxidation of dopamine is higher than that when dopamine is co-incubated with tyrosine- or serine-lacking synthetic peptide. These results suggest that dopamine related oxidative modification affect mainly Met127 in α-synuclein because of the higher local concentration of H\(_2\)O\(_2\)\(^{245}\), however the literature reports conflicting results in this regard. Whereas the results mentioned above hint at methionine oxidation as a trigger for α-synuclein aggregation, two studies reported that methionine oxidation slows the formation of fibrils\(^{246,247}\). Oxidative stress induces tyrosine nitration in α-synuclein\(^{248}\). Lewy bodies containing α-synuclein with tyrosine nitration, and nitrated α-synuclein were shown to cause the death of dopaminergic neurons in rats\(^{249,250}\). Finally, tyrosine nitration fosters the aggregation of wild-type α-synuclein\(^{251}\). Thus, a link between oxidative stress and the formation of oligomers can confidently be established. Upon exposure of recombinant α-synuclein to nitrating agents, the formation of nitrated α-synuclein oligomers stabilized by a covalent cross-link of o,o’-dityrosine occurs\(^{125}\). Upon incubation with Cu, Zn-SOD, and H\(_2\)O\(_2\), the Ala53Thr mutant oligomerizes faster. This experimental setup suggests that the generation of hydroxyl radicals mediates the oligomerization. Antioxidant molecules like carnosine and anserine prevent the oligomerization of the Ala53Thr mutant\(^{252}\).
1.3 Monohydrogen ascorbate

The potential role of antioxidants in the prevention of the formation of α-synuclein oligomers remains unexplored. Given the reductive potential of monohydrogen ascorbate (Hasc\textsuperscript{−}), we want to investigate whether Hasc\textsuperscript{−} can reduce the tyrosyl radicals in α-synuclein before oxidized α-synuclein can oligomerize. We discuss the relevant biology and chemistry of Hasc\textsuperscript{−} below.

1.3.1 Monohydrogen ascorbate (Hasc\textsuperscript{−}) and its functions in the brain

Hasc\textsuperscript{−} is essential to neural maturation\textsuperscript{253-257} and assists in neurotransmission processes. It is a key co-factor in enabling the enzyme dopamine β-hydroxylase to convert dopamine into norepinephrine, a step essential to the biosynthesis of the neuromodulator catecholamine\textsuperscript{258,259}. Studies have established that Hasc\textsuperscript{−} acts as a neuromodulator of dopamine- and glutamate-mediated neurotransmission\textsuperscript{260-265}.

Under ischemic stress, brain cells release Hasc\textsuperscript{−}\textsuperscript{266}, and neuron and glial cells take glutamate up in exchange\textsuperscript{267,268}. Lower concentrations of extracellular glutamate result in lower excitotoxicity of the brain cells after activation of glutamate receptors by Hasc\textsuperscript{−}\textsuperscript{260,261}.

The interaction in the striatum between ascorbate and dopamine is well regulated\textsuperscript{269-272}. A review outlined the importance of substantia nigra in dopaminergic and ascorbate interactions in the neostriatum, possibly through an intricate pathway of innervation and synaptic signaling\textsuperscript{260}. Hasc\textsuperscript{−} also plays a role in the regulation of both acetylcholine and catecholamine release from synaptic vesicles\textsuperscript{269,272-274}. Finally, it also takes part in the amidation of neuropeptides\textsuperscript{275} and assists in the release of these neuropeptides in the brain\textsuperscript{276,277}.

Whereas the role of Hasc\textsuperscript{−} in the synthesis of collagen is well established, it is less understood when it comes to the synthesis of collagen in the brain. Indirect evidence of the role Hasc\textsuperscript{−} plays in the synthesis of collagen in the brain is demonstrated by the fact that mice deficient in brain Hasc\textsuperscript{−} die of capillary hemorrhage in the brain penetrating vessels\textsuperscript{278}. The addition of ascorbate to a mixed culture of rat Schwann cells and dorsal root ganglion neurons enhances myelin
formation and differentiation by Schwann cells. Several experts regard this process as a result of the ability of Schwann cells to generate collagen\textsuperscript{279}.

1.3.2 Provision of monohydrogen ascorbate (Hasc\textsuperscript{–}) to neuron cells

There are two mechanisms of Hasc\textsuperscript{–} provision to neurons. The first mechanism is the active transport of Hasc\textsuperscript{–} with the sodium-dependent vitamin C transporter type 2 (SVCT2)\textsuperscript{280}. The second mechanism is the indirect provision of Hasc\textsuperscript{–} with facilitated diffusion of the bicyclic hemiketal form of dehydroascorbate to neurons\textsuperscript{281-286}. The active transport route is, however, the main entry for ascorbate into neurons\textsuperscript{287}.

Hasc\textsuperscript{–} does not enter the central nervous system by the most direct way through the blood-brain barrier. Brain endothelial cells do not express SCVT2 \textit{in vivo}\textsuperscript{288,289}. Under sufficient blood concentrations, dehydroascorbate can cross the blood-brain barrier faster than ascorbate, probably through uptake on GLUT1 in the endothelial cells\textsuperscript{290,291}. This pathway may be only an emergency route in case of the depletion of Hasc\textsuperscript{–} under oxidative conditions, because it allows for the quick recycling of Hasc\textsuperscript{–} from a higher concentration of dehydroascorbate in blood\textsuperscript{292}.

To achieve the millimolar concentrations of Hasc\textsuperscript{–} in the central nervous system, a two-step mechanism ensures a steep Hasc\textsuperscript{–} gradient from blood to neuronal cells. Hasc\textsuperscript{–} first crosses from the plasma to the cerebrospinal fluid across the epithelium of the choroid plexus\textsuperscript{293,294}. In humans, this mechanism brings the concentration of Hasc\textsuperscript{–} in cerebrospinal fluid to 0.2 mM, up from 0.05 mM in the plasma\textsuperscript{295,296}. From the cerebrospinal fluid, Hasc\textsuperscript{–} crosses to the brain interstitium by diffusion, where it achieves a concentration between 0.2 to 0.4 mM\textsuperscript{297,298}. From the interstitial space to the central nervous system, Hasc\textsuperscript{–} can either enter through active transport or through facilitated diffusion under the form of dehydroascorbate. SVCT2 is expressed at relatively high levels in the brain in comparison to other tissues\textsuperscript{278,280,299-301}. These observations make the SCVT2 route the more likely of the two routes from the brain epithelium to the central nervous system. Intracellular concentrations of neurons range between 2 to 10 mM\textsuperscript{267,302}.
1.3.3 Monohydrogen ascorbate (Hasc\(^{-}\)) as antioxidant

Hasc\(^{-}\) is acknowledged as the most efficient and biologically available antioxidant. At the molecular level, ascorbate action as an antioxidant is usually considered to be that of a PROS scavenger, and defined as “a chemical agent that prevents the oxidation of a molecule by acting as a nucleophilic reducing species that directly reacts with oxidants”. scavenger\(^{303}\). This misconception is based on the fast one-electron reactions between antioxidants and free radicals\(^{303}\), such as Hasc\(^{-}\) and hydridooxygen(\(•\)), \(k = 6.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}\)\(^{304-308}\). In this study, we follow the approach that ascorbate targets oxidized proteins to repair oxidative damage before it spreads. Therefore, we prefer the following definition of an antioxidant by Halliwell and Gutteridge: “Any substance that, when present at low concentration compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate”\(^{309}\).

In the case of neurons and Hasc\(^{-}\) as antioxidant, physiological studies hint at a possible role of ascorbate as an antioxidant. Hasc\(^{-}\) level in neurons is tenfold that of Hasc\(^{-}\) level in glia\(^{302}\). This ratio correlates well with the estimate that the rate of oxidative metabolism in neurons is tenfold higher than that of glia\(^{310}\).

In the event of cerebral ischemia, ascorbate is lost from cells into the extracellular fluid due to anoxic depolarization\(^{266}\). If the ischemia persists, the concentrations of Hasc\(^{-}\) and other low molecular weight antioxidants plummet\(^{311}\). After aerobic metabolism returns to normal, cells lack these antioxidants\(^{267}\). Hasc\(^{-}\) reduces glutamate-generated PROS and prevents subsequent cell death in cultured neurons\(^{262,263}\). In vitro experiments show that, upon exposure to oxidative stress, cultured cells and tissues have depleted concentrations of Hasc\(^{-}\)\(^{312-315}\).

The specific role of Hasc\(^{-}\) in Parkinson’s disease is ill-defined. Two independent studies reported that antioxidant levels in Parkinson’s disease were not depleted\(^{316,317}\). L-dopa (Figure 3) therapy is the standard treatment of Parkinson’s disease. Hasc\(^{-}\) plays a role in the mediation of the effects of this therapy. Studies established that neuroprotective and neurodegenerative effects of L-dopa are due to its autoxidation and the generation of radicals that result. The protection triggered by low concentrations of L-dopa results from the activation of intracellular protective mechanisms. Low-level injury induces intracellular protective mechanisms, whereas overwhelming injury
induces cytotoxicity. In the former case, ascorbate counter-balances the cellular response, in the latter case, ascorbate mitigates pro-oxidant effects of L-dopa \(^{303,318-329}\).

Although population studies show no protective effects of ascorbate to treat Parkinson’s disease \(^{330}\), a study showed that ascorbate enhances bioavailability of L-dopa. Patients with an otherwise low baseline of levodopa bioavailability at the onset of the study had their L-dopa levels increased by ascorbate treatment \(^{331}\). Ascorbate also reduces the oxidative damage resulting from MPTP administration \(^{332}\).

Hasc\(^-\) can reduce tryptophanyl and tyrosyl radicals with reaction rates ranging from 1.1 to \(8.3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}\) \(^{333}\). Hasc\(^-\) reduces tyrosyl radicals on protected tyrosine at a rate of \(2.6 \times 10^8 \text{ M}^{-1}\text{s}^{-1}\) \(^{334}\). Lysozyme tyrosyl radicals oxidize ascorbate at a rate of \(1.1 \times 10^7 \text{ M}^{-1}\text{s}^{-1}\) \(^{333}\). A study compiled the reaction rate constants for ascorbate oxidation by protein tyrosyl radicals of insulin, chymotrypsin, pepsin, and \(\beta\)-lactoglobulin. The respective rate constants are \(2.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}\), \(4 \times 10^7 \text{ M}^{-1}\text{s}^{-1}\), \(3.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}\), and \(4.0 \times 10^5 \text{ M}^{-1}\text{s}^{-1}\) \(^{334}\). This study concluded that Hasc\(^-\) loss upon oxidative stress could be due to protein repair, since protein tyrosyl radicals quickly oxidize Hasc\(^-\). The other important conclusion is that if protein tyrosyl radical reduction by Hasc\(^-\) is so fast, then the reduction of carbon-centered radicals by Hasc\(^-\) may also be competitive with the formation of harmful peroxyl radicals in proteins.

The ascorbyl radical (asc\(\bullet^-\)) has two \(pK\) values with \(pK_1 = 1.1\) and \(pK_2 = 4.3\) \(^{335}\). In comparison, the \(pK_a\) values of L-ascorbic acid are 4.0 and 11.7 \(^{336}\). In neutral solution, asc\(\bullet^-\) will disproportioniate to yield Hasc\(^-\) and dehydroascorbate \(^{335}\). At \(pH < 4\), dehydroascorbate irreversibly changes to 2,3-diketo-1-gulonic acid in a non-oxidative manner (Figure 10) \(^{337}\). Since we performed our experiments at neutral pH, we can focus our attention on
Figure 10: Monohydrogen ascorbate metabolism. Reactions and structures are shown. Adapted from Harrison et al. with permission from the rights owner.

HAsc\(^{−}\), asc\(^{−}\), and dehydroascorbate only. Bielski et al. determined that asc\(^{−}\) has an extinction coefficient at 360 nm of \(\varepsilon_{360} = 4.0 \times 10^{3} \text{ M}^{-1} \text{s}^{-1}\) and at 400 nm \(\varepsilon_{400} = 5.0 \times 10^{2} \text{ M}^{-1} \text{s}^{-1}\).
1.4 Electron transfer

1.4.1 Marcus theory

The Marcus theory provides a theoretical framework to electron transfer that was developed further over the years to account for intramolecular electron transfer in proteins and peptides. Experiments were performed by Gray, Winkler, and coworkers with proteins to define the parameters of the Marcus-Levich equation\(^{341}\). The values calculated from these experiments give us useful reference points to which we can compare our own results.

The Marcus theory is a model that relates electron transfer to the difference of electrode potentials between an electron donor and an electron acceptor. It links a kinetic parameter, the rate constant of an electron transfer \(k_{ET}^0\), to a thermodynamic parameter, the driving force \(\Delta G^0\). \(\Delta G^0\) is derived from the \(E^o\) values of the redox couples involved in electron transfer. The rate of electron transfer also depends on another parameter, the reorganization energy \(\lambda\). \(\lambda\) describes the amount of work necessary to rearrange the atoms in the outer solvent shells, that is, a change in solvent polarization, of both reactants from initial to final state, not including the influence of the charge being transferred\(^{338,339}\). The rate of electron transfer is expressed as follows:

\[
k_{ET} = k_{ET}^0 \exp \left[ -\frac{(\lambda + \Delta G^0)^2}{4\lambda R T} \right]
\]  

where \(k_{ET}\) expresses the rate of electron transfer as a function of the reorganization energy \(\lambda\) and driving force \(\Delta G^0\). \(k_{ET}^0\) is the maximum transfer rate, \(R\) is the gas constant, and \(T\) is the temperature. \(k_{ET}^0\) is obtained when \(\Delta G^0 + \lambda = 0\). For \(-\Delta G^0 > \lambda\), the rate of electron transfer decreases with increasing exothermicity. This is known as the inverted region of Marcus theory.
1.4.2 Distance dependent electron transfer

An intrinsic assumption in Marcus theory is that the electron donor D and the electron acceptor A are close to each other. Only the atoms involved in electron transfer exchange energy. To account for non-adiabatic electron transfer where D and A are separated by a distance \( r \), a correction factor has to be introduced. This factor is the determinant of a matrix that represents the electronic coupling between the initial and final electronic states, \( H_{AD} \). It is a measure for the similarity of the electronic states before and after the reaction. Equation 1 is expanded to the Marcus-Levich equation for non-adiabatic electron transfer:

\[
k_{ET} = \left( \frac{4\pi^3}{\hbar^2 k_B T} \right)^{\frac{1}{2}} |H_{AD}|^2 \exp \left( -\frac{(\Delta G^0)^2}{4k_B T} \right)
\]

(2)

where \( |H_{AD}| \) is the determinant of the matrix of electronic coupling between the initial and final state and \( k_B \) is the boltzmann constant in J/K.

The square of the determinant of \( H_{AD} \) represents the probability of an electron to tunnel through the potential barrier between D and A. The distance between these is a characteristic of this potential barrier. This distance defines \( H_{AD} \) and consequently the rate of electron transfer. The extent of electron coupling between the initial and the final state drops exponentially with the distance that separates D and A:

\[
H_{AD} = H_{AD}^0 \exp(-\beta [r_{DA} - r_0])
\]

(3)

Where \( r_{AD} \) is the actual distance between A and D, \( r_0 \) is the direct contact donor/acceptor distance, and \( \beta \) is the distance decay constant.

The distance decay constant \( \beta \) describes how the medium that separates D and A assists in electron transmission, because \( \beta \) has a value of 3-5 Å\(^{-1}\) in vacuum and 1.65 Å\(^{-1}\) in the condensed phase\(^{341}\). For \( \beta = 1.65 \) Å, an electron will tunnel, on average, 10 Å every 6 hours. Studies on active enzymes, however, reported electron transfer over distances as long as 20 Å that occur in the millisecond range\(^{342}\). This observation indicates that the peptide matrix efficiently mediates
electron transfer. Two models offer an explanation for this experimental observation: the superexchange and the electron hopping models\textsuperscript{343}.

1.4.3 Electron transfer through proteins and peptides

1.4.3.1 Superexchange model

Semiconductivity as a model for electron transfer in peptides and proteins was ruled out early on when it was realized that physiological temperatures are too low to overcome the band gap\textsuperscript{344}. A mechanism based on electron tunneling was put forth after it was observed that cytochrome oxidation in \textit{Chromatium vinosum} exhibits only poor temperature dependence\textsuperscript{345}. Hopfield described a model for intermolecular electron transfer in proteins by a thermally activated tunneling process, for which $\beta$ was estimated to be $1.44 \text{ Å}^{-1}$\textsuperscript{346}. McDonnell conceptualized the superexchange model and proposed that electrons tunnel from D to A across a bridge of n identical repeat units. The combination of the orbitals of electron donor D, of bridge units B, and of electron acceptor A, constitutes a virtual intermediate state that allows electron tunneling in a single step (Figure 11).

The associated electronic coupling matrix is interpreted as the function of the coupling between the donor and the acceptor sites and the bridge units, the coupling between bridge units, and the energy gap between the initial state of the tunneling electron and the state of the bridge when it accepts the transferred electron\textsuperscript{347}. The mixing of all available electronic states results in a lower potential barrier, which in turn leads to an increased tunneling probability $|H_{AD}|^2$, expressed by a lowered distance decay constant $\beta$. This allows for a higher electron transfer rate in comparison to the transfer rate in vacuum or the condensed phase.
An objection to this model is that peptides can be made of 21 different amino acids, which do not form the simple poly{–(\(-\text{CH}_2\)–)} bridge that McConnell used for his model. The various amino acids may impede or divert electron transfer along the bridge units. The observation that electron transfer rates measured in different types of proteins, like the centers of different photosynthetic bacteria, semisynthetic proteins, and synthetic proteins, are almost identical for comparable D-A distances prompted Dutton and coworkers to conclude that electron transfer pathways are optimized in terms of distance only. With little influence from the protein or peptide sequence, and thus the protein structure, Dutton’s model views the protein matrix as a uniform barrier with an estimate of $\beta$ as 1.4 Å\(^{-1}\)\(^{349,350}\). A refined version of Dutton’s uniform barrier model includes the packing density of the protein matrix as an additional factor in order to take into account a coarse estimate of structural differences\(^{351}\). Dutton’s model is based on the hypothesis that the
evolution of electron transfer proteins selected stable structures and protein sequences that fitted exactly this purpose. This hypothesis restricts the peptide matrix to a mostly uniform electron transfer behavior, limited by donor-acceptor distances, and ignores the properties of electron transfer in special enzymes.\[352\].

Later, experiments by Gray and Winkler put this hypothesis into question. They first selected naturally occurring metalloproteins with copper or iron cofactors that had a known protein structure. Azurins\[353\] and cytochromes\[354\] fitted the above-mentioned criteria. Based on the published structures, they attached a ruthenium complex on histidine residues at defined distances from the enzyme cofactor. The Ru\(^{2+}\) complex was photo-excited and was subsequently oxidized to Ru\(^{3+}\) by an external acceptor (or quencher) in a flash-quench manner. This initiated electron transfer from the enzyme metal cofactor to Ru\(^{3+}\). The reduced quencher then reduced the oxidized metal cofactor. The rates of electron transfer were measured by UV/Vis spectroscopy of the transient metal complexes. The durations of electron transfer measured with this method vary from sub-microseconds to seconds.\[341\]. Data collected from azurin experiments established that electron transfer occurs in accordance with the tunneling model. It takes only microseconds over distances between 15-20 Å with a distance decay constant \(\beta = 1.1 \text{ Å}^{-1}\) for a \(\beta\)-strand structure.\[341\]. However, deviations from the tunneling model were observed in many cases. Electron transfer rates for equivalent D-A distances can vary by several orders of magnitude, or electron transfer rates can be similar for D-A distance variations as high as 5 Å.\[355\]. The conclusion is that the specific structure of the protein matrix controls electron transfer rates, which contradicts Dutton’s view of the protein matrix as a uniform barrier with a fixed \(\beta\).

These observations prompted the development of a new and more complex theoretical model that includes the properties of the protein secondary structure.\[356-358\]. The underlying assumption is that covalent bonds, proton-bridges, and through-space contact offer electronic coupling pathways that can be optimized for electron transfer. Distinct coupling decay constants are assigned to each of the three elements, \(\varepsilon_c\), \(\varepsilon_H\), and \(\varepsilon_S\). The electron coupling matrix \(H_{AD}\) is then proportional to the product of all coupling constants for a given pathway:

\[
H_{AD} \propto \Pi \varepsilon_c \Pi \varepsilon_H \Pi \varepsilon_S
\]
where $\epsilon_c$ is the product of covalent bonds coupling decay constant, $\epsilon_H$ is the proton-bridge coupling decay constant, and $\epsilon_S$ is the through-space coupling decay constant. Protein structure data provides the necessary input for computational search for the optimal pathway. This approach provides good agreement between theoretical electron transfer rates and measured ones in modified metalloproteins\textsuperscript{357}. The model also allows the prediction of the electron transfer features of secondary structure motifs. Further improvement of the model includes protein dynamics\textsuperscript{342,359-361}.

In short, protein folding is the key determinant of the superexchange pathway model, whereas the uniform barrier model considers protein folding as negligible. Recent research results support the former model\textsuperscript{362,363}, which accounts for the observed variations in the distance decay constant $\beta$ by allowing modulation in electronic coupling through covalent bonds, hydrogen bonds, and through-space.

### 1.4.3.2 Hopping

The naturally small driving forces available for electron transfer constrain the limit over which the superexchange mechanism is effective. Dutton estimates that the maximum distance for electron transfer is 14 Å\textsuperscript{350}, however Gray and Winkler regard it to be 20 Å\textsuperscript{341}. Dutton proposed that a mechanism known as electron hopping could explain electron transfer in enzymes in which the effective transfer distance exceeds 14 Å. According to Dutton, the actual alignment of metal-containing redox-cofactors, particularly Fe-S-clusters and hemes, can explain electron transfer cascades in enzymes\textsuperscript{350}.

DNA transports charges with an efficiency proportional to the number of guanine pairs available, this concept served as the experimental basis to develop the hopping model. Superexchange theory failed to explain the large amount of experimental data collected on electron transfer through DNA double strands\textsuperscript{364-368}. The $\beta$ distance decay constant values expected for an organic matrix like DNA range between 0.6 and 1.2 Å\textsuperscript{-1}, much too low to explain the long distance electron transfer in DNA. The guanine base pairs, however, have a low electrode potential for their redox couple with the corresponding radicals. Thus they serve as short distance stepping-stones for electron transfer through the creation of oxidized chemical intermediates. As a result,
the electron transfer rate increases proportional to the logarithm of the number of involved base pairs, thus changing the dependence of D-A distance of the superexchange theory. The hopping model is based on\textsuperscript{369-371}:

\[ k_{ET} \propto k_{IB} n^{-\eta} \]  

(5)

where \( k_{ET} \) is the rate constant of electron transfer, \( k_{IB} \) is the interbase electron transfer rate, and \( n \) is the number of base pairs involved. \( \eta \) has values between 1 and 2.

The hopping theory was confirmed experimentally in studies of Giese and coworkers\textsuperscript{372}.

The formation of chemical intermediates, either oxidized or reduced relays \( R \), is the mechanistic backbone for the sequential electron transfer. Localized relay sites \( R \) form a bridge for the electron transfer, at the start of which an electron donor \( D \) gives an electron to the first relay site, which then transfers the electron further until the last relay site donates the electron to the electron acceptor \( A \) (Figure 12). As predicted by thermodynamics, endergonic transfer steps may occur. It is also possible that the sequence starts with an electron transfer from a bridge element to the acceptor\textsuperscript{350,373}. 
Intramolecular electron transfer in peptides can also occur, albeit the exact electron pathway is not yet established. One model proposes that peptide bonds may act as relays for electron transfer, that is, peptide dynamics are a key parameter. Calculations show that energy barriers for electron transfer across adjacent residues are correlated to the Ramachandran angles $\Phi$ and $\Psi$. From the theory we expect that the arrangement of adjacent peptide bonds into a favorable angle minimize the distance between neighboring carboxyl groups to 2.8 Å. This geometry allows the fast sequential redox reactions across a peptide chain.

The presence of oxidizable aromatic residues is not required for electron hopping to occur. It has been shown that an electron is transferred from a pyrimidine ruthenium complex $\text{Ru(II)(bpy)}_2$ to an amine ruthenium complex $\text{Ru(III)(NH}_3)_5$ linked by an oligopeptide made up of proline residues only. The determination of differences in the rate as a function of the number of proline linkers revealed that a mechanistic transition from a superexchange model (exponential distance
dependence) to hopping (linear distance dependence) occurs when the number of proline linkers exceeds five.\textsuperscript{377}

Electron transfer through self-assembled monolayers of oligoglycine also exhibits linear distance dependence.\textsuperscript{378,379} One report states that an oxidized tyrosine residue in Photosystem II causes perturbation in FT-IR spectrum attributed to spin delocalization on adjacent peptide bonds. The authors of the study surmised that the small delocalization indicates a role of the peptide bond in electron transfer.\textsuperscript{380}

All these experimental results about the involvement of the peptide bond in electron transfer through a peptide defy the established wisdom. The oxidation of peptide bonds to form intermediates in electron hopping to facilitate electron transfer appears unlikely due to the high activation energy necessary for such a reaction to occur.\textsuperscript{341} An alternative to the necessary electron coupling provided by bridging residues between D and A for hopping models is through space electron transfer. In this model, electron transfer occurs either directly from D to A through orbital overlap, or with the involvement of solvent molecules.\textsuperscript{381} Experiments on model peptides with proline spacers showed that rates of electron transfer do not depend on the number of spacers. This suggests that through space electron transfer is the favored pathway.\textsuperscript{382-384}

**1.4.4 Electron transfer between oxidized methionine and tyrosine**

**1.4.4.1 Electrode potentials of methionine and tyrosine**

Tyrosyl radical has a life span too short to allow the determination of its electrode potential by linear sweep voltammetry. The method of choice is to measure the electrode potential of a phenoxy radical against a reference compound by pulse radiolysis, if there is a rapid equilibration between the two. Either the equilibrium concentrations are determined or the rate constants of forward and backward reactions are measured.\textsuperscript{385,386} A limitation to the latter approach is that corresponding measurements have to be carried out at pH \( \geq 11 \), because the electron transfer from phenolates is orders of magnitude faster than that of phenols. The electrode potential of tyrosine ranges from 0.71 to 0.74 V with phenol as a reference in a pH range of 11-12.\textsuperscript{386,388} With Osmium(II)terpyridine_2 as a reference, the electrode potential was
measured to be $E^\circ = 0.76$ V at pH 11 389. The corrected electrode potential at pH 7.0 is $E^{\circ'} = 0.99$ V. Faraggi et al. measured the electrode potential at pH 7 to be $E^{\circ'} = 0.93$ V 390.

The electrode potential of methionine is evaluated from the peak potential $E_p$(Met/Met$^{\bullet+}$) $\geq$ 1.3 V 391. At pH 1, methioninyl radical cation decays into $\alpha$ (thioalkyl) alkyl radical with a half-life of $t_{1/2} = 5$ µs 392. Equilibration reactions in pulse radiolysis are therefore too slow to measure $E^\circ$(Met/S$^{\bullet+}$/Met). Prütz et al., however, estimated the reduction potential at pH 6.8 of the species Met/S$\vdash$N$^+$ to be $E^\circ$(Met/S$\vdash$N$^+$/Met) = 1.4 V393 with the electrode potential $E^\circ$(N$_3^-$/N$_3^-$) = 1.3 V394 as a reference. It is clear from these electrode potential estimates that the oxidation of tyrosine by oxidized methionine is feasible.

1.4.4.2 The generation of tyrosine and methionine radicals

The reaction HO$^\bullet +$ Met has a pH independent rate constant of $8.1 \times 10^9$ M$^{-1}$ s$^{-1}$ 395,396. The reaction HO$^\bullet +$ Tyr has an average rate constant of $1.3 \times 10^{10}$ M$^{-1}$ s$^{-1}$ 397-399 at neutral pH.

In the case of tyrosine, HO$^\bullet$ can either oxidize tyrosine to the corresponding tyrosyl radical (TyrO$^\bullet$) or form an adduct with the aromatic ring either on the meta position ($m$-TyrOH-OH$^\bullet$) or on the ortho position ($o$-TyrOH-OH$^\bullet$), which both form TyrO$^\bullet$ by elimination of water. The ratio between these two reactions is 5:85 (Figure 13)399. The remaining unidentified reaction spots of HO$^\bullet$ on tyrosine cannot be attributed with certainty to any chemical species. The identifiable species have estimated extinction coefficients of $\varepsilon_305(m$-TyrOH-OH$^\bullet$) = $2.8 \times 10^3$ cm$^{-1}$M$^{-1}$, $\varepsilon_{330}(o$-TyrOH-OH$^\bullet$) = $3.0 \times 10^3$ cm$^{-1}$M$^{-1}$, $\varepsilon_{260}$(TyrO$^\bullet$) = $6.0 \times 10^3$ cm$^{-1}$M$^{-1}$, and $\varepsilon_{405}$(TyrO$^\bullet$) = [2.0-3.2] $\times 10^3$ cm$^{-1}$M$^{-1}$ 399-401.
Upon its reaction with methionine, HO' can either form a hydroxysulfanyl radical adduct with the thioether moiety, or directly oxidize the methionine to a α-(alkylthio) alkyl radical on either Cγ or Cε. The ratio between the former and the latter reactions is 80:20. The former reaction leads to the formation of the sulfur radical cation Met/S⁺⁺, which subsequently deprotonates to a α-(alkylthio) alkyl radical. The sulfur radical cation Met/S⁺⁺ can be stabilized by forming an intramolecular two center-three electron bond Met/S:.N⁺ or Met/S:.O.
This two center-three electron bond could either be formed with nitrogen or oxygen atoms of a peptide bond or from a carboxylic group. The species described above have the following extinction coefficients: \( \epsilon_{290} (\text{Met/S}^+) = 3.0 \times 10^3 \text{ cm}^{-1}\text{M}^{-1} \), \( \epsilon_{385} (\text{Met/S}:+\text{N}^+) = 4.5 \times 10^3 \text{ cm}^{-1}\text{M}^{-1} \), and \( \epsilon_{385} (\text{Met/S}:+\text{O}^+) = 4.5 \times 10^3 \text{ cm}^{-1}\text{M}^{-1} \). If the concentration of unprotected methionine is higher than 0.1 mM, this leads to the formation of Met\(_2\)/S+:S\(^+\) species, which absorbs strongly between \( \lambda = 350 \text{ nm} \) and \( \lambda = 600 \text{ nm} \). 

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\[^{402}\text{References}\]
Figure 14: Reaction scheme for the reaction Met + HO$^\cdot$

1.4.4.3 Evidence of oxidation of tyrosine by oxidized methionine

Methionine, after the formation of a $\sigma - \sigma^*$ bond between methionine and Br$^-$ Met/S$^\cdot$Br$^-$, intramolecularly oxidizes tyrosine in a peptide$^{407,408}$. The species Met/S$^\cdot$Br$^-$ oxidizes tyrosine with a rate constant of $k = 1.1 \times 10^5$ s$^{-1}$ in Met$^5$-enkephalin$^{409}$. Methionine was shown to be an
efficient relay amino acid of electron transfer from a dialkoxyphenylalanine residue to a tyrosine residue across a poly-proline\textsuperscript{410}.

Can an methionine radical in an appropriate protein environment oxidize a neighboring tyrosine? This question was addressed in several studies. One of these showed that methionine in the protein calmodulin forms a Met/S.$\cdot$.N\textsuperscript{+} species by the interaction of the sulfur atom of methionine with the nitrogen of the peptide bond. Met/ S.$\cdot$.N\textsuperscript{+} oxidizes a neighboring tyrosine\textsuperscript{411}. \textit{Ab initio} calculations hint at the involvement of a S.$\cdot$.\pi bond formation between methionine and aromatic residues that would allow methionine to participate in the protein electron hole transport. S.$\cdot$.\pi formation can decrease the local ionization energies and its binding energy is low enough that protein vibrations can dissociate this bond to enhance electron hole relay\textsuperscript{412}. The anodic peak potential of the 6-\textit{endo}-\textit{(Methylthio)bicycle[2.2.1]heptane-2-\textit{endo}-carboxamide} compound is 0.33 V lower than that of 6-\textit{endo}-\textit{(Methylthio)bicycle[2.2.1]heptane-2-\textit{exo}-carboxamide} (Figure 15)\textsuperscript{413}, a clear indication that there is a S.$\cdot$.\pi bond that forms between a phenyl group and a thioether group.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{norbornane.png}
\caption{Norbornane species used by Chung \textit{et al.} in 2009.}
\end{figure}

\textit{A}; 6-\textit{endo}-\textit{(Methylthio)bicycle[2.2.1]heptane-2-\textit{exo}-carboxamide.}

\textit{B}; 6-\textit{endo}-\textit{(Methylthio)bicycle[2.2.1]heptane-2-\textit{endo}-carboxamide}
The first direct observation of this S:π bond was obtained by photoelectron spectroscopy. The photoelectron spectrum of 6-endomethylthio-2-endoaryl bicyclo[2.2.1]heptane clearly shows the contribution of a S:π bond\textsuperscript{414}. Finally, oxidized methionine in met-enkephalin is reduced by tyrosine, which results in the formation of a tyrosyl radical that eventually leads to the formation of a tyrosine dimer\textsuperscript{415}. 
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Chapter 2 MATERIALS AND METHODS

2.1 Expression and Purification of α–synuclein

2.1.1 Chemicals

Molecular biology grade standard reagents were obtained from Sigma-Aldrich Co. (St. Louis, USA), FLUKA AG (Buchs, Switzerland), and MERCK (Zug, Switzerland). Ampicillin and isopropyl β–D–thiogalactopyranoside were obtained from AppliChem. Ultrapure water was prepared with a Merck Millipore (Zug, Switzerland) Advantage device.

A HiPrep Q FF 16/10 column and a HiPrep Phenyl FF 16/10 column from GE Healthcare Life Science were used for anion exchange chromatography and hydrophobic interaction chromatography, respectively. For reverse–phase high–performance liquid chromatography analysis of α–synuclein purity, a Discovery BIO C18 wide pore column from Sigma–Aldrich Co. was used.

A dialysis membrane with a 3.5 kDa molecular weight cut–off was purchased from Merck Millipore (Zug, Switzerland) for overnight buffer exchange by dialysis tubing. A dialysis membrane with a 5 kDa molecular weight cut–off was purchased from Merck Millipore (Zug, Switzerland) for the buffer exchange in an Amicon stirred cell for pressure-based solute concentration. The Amicon stirred cell was purchased from Merck Millipore (Zug, Switzerland).

We used E. coli BL21 Star DE3 cells from Life Technologies. We transformed the BL21 Star DE3 cells with the pRK172 plasmid carrying the wild–type human gene α–synuclein (kindly donated by Dr M. Goedert, Medical Research Council Laboratory of Molecular Biology, University of Cambridge, England).
2.1.2 Expression of α–synuclein

A new plasmid transformation was performed for each batch, namely BL21 Star DE3 cells were transformed by applying heat shock. The transformed cells were then incubated in lysogeny broth for 60 min at 37°C. Then the transformed cells were plated on a lysogeny broth–plate and incubated overnight at 37°C. The following day, the Petri dishes were washed with 3 mL of lysogeny broth. Then 100 mL of lysogeny broth, which contained 0.1 g/L ampicillin, was incubated with the 3 mL lysogeny broth wash. The cells were then grown at 37°C under vigorous shaking until OD_{600} reached a value of about 1.0. Following this, 1 L of lysogeny broth, which contained 0.1 g/L ampicillin, was inoculated with 25 mL of the cell culture. The cells were then grown at 37°C under vigorous shaking until OD_{600} reached a value of about 1.0. The protein expression was then initiated by the addition of 1mL of 1.0 M isopropyl β–D–thiogalactopyranoside. The protein expression was carried out for 4 hours at 37°C under vigorous shaking. We separated the cells from the medium by centrifugation at 6000 rpm at 4°C.

2.1.3 Cell lysis and purification of wild–type α–synuclein

The periplasmic extraction method 1 was used to purify the α–synuclein proteins present in the cell periplasm. The purification of the periplasmic extract was performed with a non–denaturing protocol. This protocol allows high yields of recombinant α–synuclein (80–90 mg per liter of lysogenic broth) with 98% purity as determined by reverse–phase high–performance liquid chromatography (RP–HPLC).

The cell pellet was resuspended and carefully homogenized in an osmotic shock buffer (40% sucrose, 30mM Tris–HCl, 2mM ethylenediaminetetraacetate, pH 7.2) and incubated for 10 min at room temperature. The pellet was collected by centrifugation at 12 000 rpm for 20 min at 4°C. After discarding the osmotic shock buffer, the pellet was resuspended quickly with 90 mL of a solution of cold water and 37.5 µg of MgCl₂. The solution was kept on ice for 3 min. The supernatant containing the periplasmic proteins was collected by centrifugation at 12 000 rpm for 20 min. The supernatant was filtered on filter paper after addition of Tris–HCl to obtain an
end concentration of Tris–HCl of 20 mM. Then the filtrate was loaded onto a chromatography column and eluted by anion exchange chromatography with a 0-0.5 M NaCl gradient in 20 mM Tris–HCl at 4°C. The α–synuclein–containing fractions (see 2.1.4 Protein characterization below) were pooled and dialyzed in a MerckMillipore dialysis membrane with a 3.5 kDa molecular weight cut–off in 20 mM bis–Tris buffer pH 8.0 with 1 M ammonium sulfate. Dialysis was performed under gentle stirring overnight at 4°C against 50 mM bis–Tris buffer at pH 7 with 1 M ammonium sulfate. Next the dialyzed solution was loaded onto a column and eluted against 50 mM bis-Tris buffer at pH 7 to remove impurities by hydrophobic interaction chromatography. The α–synuclein–containing fractions were pooled and exchanged six times against ultrapure water with an Amicon stirred cell of 200 mL and a membrane with a 5 kDa molecular weight cut–off. α–Synuclein was then divided in aliquots, lyophilized, and stored at –20°C.

2.1.4 Protein characterization

The elution fractions of α–synuclein were analyzed by SDS PAGE electrophoresis. SDS PAGE separates proteins according to their size. First, the protein is thermally denatured under reducing conditions. The protein unfolds and becomes covered with the highly negatively charged SDS molecules. When the protein is loaded onto a gel matrix and placed in an electric field, the negatively charged molecules migrate towards the positively charged electrode and are separated by molecular sieving. Only fractions that exclusively contained a band that corresponded to 19 kDa were selected. The concentration of α–synuclein was derived based on the absorptivity at λ = 280 nm. The reference extinction coefficient was calculated to be ε_{280} = 5960 M⁻¹ cm⁻¹, with the Protparam tool available from www.web.expasy.org. The calculation was based on the evaluation method developed by Edelhoch et al. and the extinction coefficients of tyrosine measured by Pace et al. ².

2.2 Experimental methods
2.2.1 Chemicals

All the synthetic peptides, which are certified to be at least 95% pure, were purchased from BIOMATIK (Cambridge, Canada). All chemicals are of analytical grade or higher and were purchased from Sigma–Aldrich Co. (St. Louis, USA) or FLUKA AG (Buchs, Switzerland). In experiments with monohydrogen ascorbate (Chapter 4), at least a 99.95% trace metals basis of sodium phosphate monobasic anhydrous and sodium phosphate dibasic anhydrous was used. Ultrapure water was prepared with a Merck Millipore (Zug, Switzerland) Advantage device.

2.2.2 Pulse radiolysis

Pulse radiolysis was used to deposit 2 MeV electrons into the solution. The physics of the processes of the energy deposition is described elsewhere\(^4\).

The energy of the electron is not deposited homogeneously; more energy is lost the slower the particle moves. Thus, most radicals are generated near the end of the track, a few mm after entering the solution. This results in a non–homogeneous distribution of products (reactions 1 and 2). Beyond 100 ns, the solution becomes homogeneous through diffusion and the kinetics can be analyzed. Ionization results in water radical cations and electrons. The radical cation of water is strongly acidic and immediately decays into a proton and hydriodooxygen(•) (HO\(^\cdot\)) (reaction 3). Water solvates the electron produced by the ionization (reaction 4). The electronically excited water molecule decays into hydriodooxygen(•) and monohydrogen(•)\(^5\) (reaction 5).

\[
\begin{align*}
    \text{H}_2\text{O} & \rightleftharpoons \text{H}_2\text{O}^\cdot + e^- \quad 1 \\
    \text{H}_2\text{O} & \rightleftharpoons \text{H}_2\text{O}^* \quad 2 \\
    \text{H}_2\text{O}^\cdot & \rightarrow \text{H}^\cdot + \text{HO}^\cdot \quad 3 \\
    e^- + n\text{H}_2\text{O} & \rightarrow e_{\text{aq}}^- \quad 4 \\
    \text{H}_2\text{O}^* & \rightarrow \text{H}^\cdot + \text{HO}^\cdot \quad 5
\end{align*}
\]
A part of the initial products HO’, H’, and eaq−, react further along the track of the fast electron and form HO−, H2O, H2O2, and H2 5 (Reactions 6, 7, 8, 9, and 10). These are known as the primary products of water radiolysis.

\[
\begin{align*}
\text{HO’} + \text{eaq}^- & \rightarrow \text{HO}^- \quad 6 \\
\text{HO’} + \text{H}^+ & \rightarrow \text{H}_2\text{O} \quad 7 \\
2\text{HO’} & \rightarrow \text{H}_2\text{O}_2 \quad 8 \\
2\text{H}^+ & \rightarrow \text{H}_2 \quad 9 \\
2\text{H}^+ + 2\text{eaq}^- & \rightarrow \text{H}_2 \quad 10 \\
\end{align*}
\]

The overall reaction of water radiolysis is shown in reaction 11. Each primary generated species has a corresponding yield: \( G(\text{eaq}^-) = 2.65, \ G(\text{HO’}) = 2.65, \ G(\text{H}^+) = 0.54, \) and \( G(\text{H}_2\text{O}_2) = 0.54 \) where \( G \) is the radiochemical yield, expressed either as molecules produced per 100 eV absorbed energy or as 0.1036 µmol generated species per 1 J/kg absorbed energy6.

\[
\text{H}_2\text{O} \Leftrightarrow \text{eaq}^- + \text{H}^+ + \text{HO}’ + \text{H}^+ + \text{H}_2 + \text{H}_2\text{O}_2 \quad 11
\]

The reaction conditions were chosen such that either hydridooxygen(•) or trinitrogen(2N-N)(•) were produced exclusively. In order to do so, we scavenged aqueous electrons with N2O to produce hydridooxygen(•), (Reaction 11). The concentration of N2O at saturation is 24 mM5.

\[
\begin{align*}
\text{eaq}^- + \text{N}_2\text{O} & \rightarrow \text{N}_2\text{O}^- \quad 11 \\
\text{N}_2\text{O}^- + \text{H}_2\text{O} & \rightarrow \text{N}_2 + \text{HO}^- + \text{HO}’ \quad 12 \\
\text{HO’} + \text{N}_3^- & \rightarrow \text{HO}^- + \text{N}_3’ \quad 13 \\
\end{align*}
\]

Some of our experiments required the exclusive production of tyrosyl radicals. In order for trinitrogen(2N-N)(•) to target tyrosine for oxidation, 0.1 M of sodium azide was added to the solution. The competition between tryptophan and tyrosine for trinitrogen(2N-N)(•) could be ignored because the synthetic peptides used in our experiments and α-synuclein do not contain tryptophan.
2.2.2.1 Experimental setup

Experiments were carried out at room temperature with a 2 MeV Febetron 705 accelerator manufactured by Field Emission Corp., presently L–3 Communications (San Leandro, CA). The accelerator delivers pulses of less than 50 ns duration with a maximum dose of 200 Gy. We adjusted the dose with an aluminum shield with holes calibrated to let the desired dose reach the sample cell. We used a custom-built 6.0 cm quartz measurement cell from Hellma (Müllheim, Germany). A DHPCA–100 amplifier from FEMTO Messtechnik GmbH (Berlin, Germany) amplified the transmission signal of a Hamamatsu R928 photomultiplier. A Yokogawa DL7100 digital storage oscilloscope digitalized the transmission signal. We used a grating of 150 grooves/mm with a 300 nm blazing. We set the monochromator slit–width to 0.4 mm.

In the section below we will address an issue pertaining to our measurements of tyrosyl radical formation. Tyrosyl radicals exhibit a very sharp band at 405 nm that is commonly used for quantification. This band posed a problem for our experiments. The maximum signal to noise ratio of an ideal absorption measurement is limited by physics:

$$\left( \frac{S}{N} \right)^2 \sim \frac{\text{number of photons}}{\Delta t}$$

Where S is the signal, N is the noise, and $\Delta t$ is the integration time per measurement point.

Therefore, the signal to noise in a practical pulse radiolysis measurement is critically dependent both on the time-resolution and the light intensity on the detector. The light sources we used emit “white” light; Xe-arc lamps with a high intensity were chosen. A monochromator selects a band of this light for analysis. The higher the optical resolution, the narrower the band, and the lower the intensity of the light reaching the detector. Therefore, the signal–to–noise ratio deteriorates with increasing optical resolution. The conditions of an experiment are thus a compromise between optical resolution, temporal resolution, and signal–to–noise ratio. For our experiments, we chose an optical band–width of > 5nm. As a consequence, we effectively measure the average of the absorption over a band–width broader than that of tyrosyl radical at $\lambda = 405$ nm. This means that the effective molar absorptivity coefficient of the tyrosyl radical in our
measurements does not correspond to the molar absorptivity coefficients published in the literature.

2.2.2.2 Dosimetry

Absolute doses were determined with KCNS dosimeter to calibrate a relative dosimeter. The latter was a coil that measured the current induced by the electron beam.

The KCNS dosimeter works as follows: in a solution of KCNS saturated with N₂O, the product (SCN)₂⁻ is formed by the reaction of KSCN with HO’. The absorbance vs. time signal of (SCN)₂⁻ was monitored at λ = 472 (Reactions 13 and 14).

\[ \text{HO'} + \text{SCN}^- \rightarrow \text{HO}^- + \text{SCN}' \]  
\[ \text{SCN}' + \text{SCN}^- \rightarrow (\text{SCN})_2^{2-} \]

The [Fe(CN)₆]⁴⁻ dosimeter works as follows: in a solution of [Fe(CN)₆]⁴⁻ saturated with N₂O, Fe(II) is oxidized to Fe(III) by HO’ as shown in reaction 15. The absorbance vs. time signal of [Fe(CN)₆]³⁻ was monitored at λ = 420 nm.

\[ \text{HO'} + [\text{Fe(CN)}_6]^{4-} \rightarrow \text{HO}^- + [\text{Fe(CN)}_6]^{3-} \]

We calculated the dose per pulse with equation 1:

\[ \text{Dose (Gy)} = \frac{A}{\varepsilon \times G \times d \times 0.1036 \mu\text{mol} \times \rho} \]  

Where dose is the absorbed energy per mass (1 Gy = 1 J/kg), A is the absorbance in AU, \( \varepsilon \) is the extinction coefficient in M⁻¹ cm⁻¹, \( \rho \) is the density of the medium, and \( d = 6.0 \) cm is the path length of the optical cell.

The extinction coefficient of (SCN)₂⁻ at 472 nm is \( \varepsilon_{472\text{ nm}} = 7.6 \times 10^3 \) M⁻¹ cm⁻¹. Under N₂O saturated conditions and [KSCN] = 10 mM, G = 6.13 is the radiochemical yield of (SCN)₂⁻.
The extinction coefficient of $[\text{Fe(CN)}_6]^{3-}$ at 420 nm is $\varepsilon_{420\text{ nm}} = 1.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. Under saturated conditions and $[\text{Fe(CN)}_6]^{4-} = 10 \text{ mM}$, $G = 6.13$ is the radiochemical yield of $[\text{Fe(CN)}_6]^{3-}$.

2.2.2.3 Solution preparation for Pulse Radiolysis Experiments

All experiments were carried out at room temperature. The solution preparation differed depending on whether the solution was to contain a synthetic peptide or $\alpha$–synuclein.

In the case of a peptide solution, aliquots of lyophilized synthetic peptides were dissolved to obtain stock solutions. An anaerobic, buffered solution was prepared inside a N₂–filled glove box. This solution was transferred to a schlenk tube, de–aerated under vacuum and saturated with N₂O; this cycle was repeated five times.

In the case of $\alpha$–synuclein, it was established that direct de–aeration of the solution led to the formation of a light–absorbent product, probably due to the denaturation of $\alpha$–synuclein. In order to prevent denaturation, a different procedure was devised.

The buffer solution was de–aerated under vacuum and saturated with N₂O five times in a separate schlenk tube connected to a pear–shaped flask with a double neck by a PEEK capillary ($\Theta$ 0.75 mM, IDEX Health and Science LLC., Oak Harbor, USA). The pear–shaped flask contained the lyophilized $\alpha$–synuclein. After the last de–aeration process of the solution under vacuum, the PEEK capillary was dipped into the N₂O saturated solution. In order to push the buffer solution through the PEEK capillary into the pear–shaped flask, the schlenk tube was filled with N₂O until atmospheric pressure was achieved.

The solution was then transferred to an air–tight syringe (10 mL, Hamilton Sample Lock). A second air–tight syringe was filled with the corresponding N₂O saturated buffer solution. For experiments with monohydrogen ascorbate, the second syringe was filled with N₂O saturated buffer solution with monohydrogen ascorbate at a concentration double that of the final concentration. The two air–tight syringes were then placed in a syringe pump prior to pulse irradiation. The solution and the buffer solution were then conveyed in parallel by peek capillaries ($\Theta$ 0.75 mM, IDEX Health and Science LLC., Oak Harbor, USA) and mixed in a
PEEK Low Pressure Tee Assembly (IDEX Health and Science LLC., Oak Harbor, USA) before it reached the irradiation cell.

The buffer solution was 5 mM phosphate, pH 7, unless stated otherwise.

2.3 Linear Sweep Voltammetry

2.3.1 Experimental setup

Cyclic voltammograms were recorded with a PGSTAT 128N (Metrohm Autolab, Utrecht, NL) integrated potentiostat/function generator/digitizer. A 33 µm carbon micro electrode (ALS Co., Tokyo, Japan) or a 2 mm glassy carbon electrode (Metrohm, Herisau, Switzerland) were used as working electrodes. The reference electrode was an Ag/AgCl (3 M KCl) electrode (Metrohm, Herisau, Switzerland). The reference electrode was regularly washed and refilled with fresh 3 M KCl to ensure a proper reference voltage. Experiments were performed at a scan rate of 1 V/s unless stated otherwise.

2.3.2 Solution preparation

Aliquots of synthetic peptides were dissolved to obtain a final concentration of 1.0 mM. The solutions were homogenized and de-aerated with a flow of N₂ for 30 seconds between each measurement. The pH values in the experimental solutions were determined after voltammetry by potentiometric measurement with a glass electrode. The buffer solutions used were the following; at pH 0.8, the buffer was 0.18 M H₂SO₄, at pH 3.8, the buffer was 0.5 mM H₂SO₄ and 0.18 M K₂SO₄, at pH 7.4, the buffer was 0.09 M KH₂PO₄ and 0.28 M K₂HPO₄. The ionic strength in these solutions was about 0.6 M
2.4 UV–Vis spectroscopy

UV-Vis spectroscopy is either absorption or reflectance spectroscopy in the UV-Vis region of the electromagnetic spectrum. The absorption of UV or visible radiation results in the excitation of outer electrons. This excitation causes three kinds of electronic transitions:

1) Transitions of $\pi$, $\sigma$, and $n$ electrons to corresponding antibonding states,

2) Transitions of electrons between atoms or groups of atoms: charge transfer,

3) Transition of $d$ and $f$ electrons between different $d$ or $f$ states.

This technique is used to determine concentrations of absorbing species in solution by applying the Beer-Lambert law (Equation 2).

$$A = \varepsilon \times b \times c$$  \hspace{1cm} (2)

Where A is the absorbance, $\varepsilon$ is molar absorptivity coefficient in M$^{-1}$ cm$^{-1}$; b is cell path length in cm, and c is the concentration of solution in M. UV-Vis spectra were recorded with SPECORD 250 (Analytik Jena AG, Jena, Germany) at room temperature.

The buffer solutions used for the recording the spectra of Ac-Tyr-NH$_2$ and Ac-AYEMPSE-NH$_2$ were the following: at pH 9.4, 13 mM borax and 7 mM sodium hydroxide, at pH 11.4, 25 mM monosodium phosphate and 18 mM sodium hydroxide.

2.5 Analysis and Simulations

2.5.1 Fitting of rate equations

Absorbance vs. time traces obtained by pulse radiolysis were fitted either with first–order, second–order, or parallel first–order and second–order rate equations with the Curve Fitting Toolbox of MATLAB® (Mathworks Inc., Natick, United States). In order to confirm the validity
of the rate equations that were fitted, the Chemical Kinetics Simulator program (IBM) was applied to simulate the formation of products. Then the results obtained from the simulations to absorbance vs. time traces were fitted by applying the extinction coefficient and cell length factors to the concentrations obtained from the simulation.

2.5.2 Estimation of the standard error

The standard error was estimated using the Student’s distribution. First the standard deviation was calculated from the residuals between fit and data and then the standard error (SE) at an interval of confidence of \( \alpha = 0.05 \) using equation 3:

\[
SE = \frac{2t_{\alpha=0.025} \cdot S}{\sqrt{v}}
\]  

(3)

Where SE is the standard error. \( t_{\alpha=0.025} \) is the cumulative t–value for the interval of confidence of \( \alpha = 0.025 \) for the degree of freedom \( v \).

2.5.3 Matsuda equation

Comparison of experimental i(E) traces with a curve obtained with the Matsuda equation for reversible cases \(^{10}\) allows for the evaluation of two features of the reactions taking place at the electrode surface. The first feature is the degree of reaction reversibility and the second is the kind of mass transport that takes place during the reaction, either controlled by diffusion or adsorption.

The Matsuda equation is an application of the Butler–Volmer equation in context of linear sweep voltammetry in a solution a rest. Further requisite conditions include the potential scan rate and the reactants’ diffusion rates. Matsuda’s generic approach starts by defining a simplified current function that substitutes all constant parameters of the experiment\(^{10}\). The Euler–McLaurin method is used to convert the simplified current function (containing an integral lacking an explicit solution) into an infinite sum with the discrete increment \( lh \). The discrete increment \( lh \)
combines potential and diffusion in time for \( v=\text{constant} \). The result is the dimensionless current function \( \psi_r(\xi) \) in equation 4:

\[
\psi_r(\xi) = \frac{h}{2\sqrt{\pi}} \left\{ \frac{1}{2} \frac{1}{\cosh^2(\xi/2)} + \sum_{l=1}^{\infty} \frac{1}{\cosh^2\left(\frac{\xi}{2l} - (l \pi)^2\right)} \right\}
\]  

(4)

Time dependence is introduced with the variability of \( E \) in time and the solution of Fick’s law for planar diffusion:

\[
\xi = -\frac{nF}{RT} [E(t) - E_{1/2}] = \frac{nF}{RT} \nu t - a
\]

(5)

\[
a = \frac{nF}{RT} (E_0 - E_{1/2}) = \ln \frac{C_{ox}}{C_{red}} \cdot D_{ox} \sqrt{D_{red}}
\]

(6)

Equation 5 defines the parameter \( \xi \), where \( E(t) \) is the potential at the electrode at time \( t \), and \( E_{1/2} \approx E_0' \) is the reversible half–wave potential that would be obtained in a polarographic experiment. The parameter \( a \) of equation 5 is expressed in equation 6, where \( D_{ox} \) and \( D_{red} \) are the diffusion coefficients of the oxidized and the reduced species at the electrode surface in m/s, respectively, and \( \nu \) is the scan rate in V/s. \( C_{ox} \) and \( C_{red} \) are the bulk concentrations of the oxidized and reduced species in solution, respectively.

Equation 4 was subsequently used to compute \( \psi_r(\xi) \) by selecting a sufficiently small \( h \) and a sufficiently large \( l_{\text{max}} \). \( \psi_r(\xi) \) can be transformed to \( j(E) \), which equals \( j(t) \) if \( v=\text{constant} \).
2.6 References

Chapter 3 REVERSIBLE ELECTRON TRANSFER FROM TYROSINE 125 TO OXIDIZED METHIONINE 127

3.1 Introduction

Parkinson’s disease is the most frequent neurodegenerative movement disorder with a worldwide estimate of 4 million affected patients, mostly elderly people, which amounts to 5% of people aged 60 and above. The symptoms are difficult to alleviate. Therefore, the stakes to understand the disease better are high. Parkinson’s disease is linked to mitochondrial complex I deficiency and subsequent cellular oxidative stress conditions. α-Synuclein is a protein central to the disease etiology, chiefly due to its propensity to form fibrils called Lewy bodies, a hallmark of Parkinson’s disease. An interesting comparison is that of α-synuclein and β-synuclein. Although the two proteins are classified in the same protein family and are 59% homologous, the protein β-synuclein has a lower propensity to form fibrils.

The oligomers of α-synuclein that act as initial seed for fibril formation are stabilized by the formation of dityrosine bonds. α-Synuclein sequence at the C-terminal domain has a cluster of three tyrosine residues. Met127 is 15 Å away from Tyr125, as calculated from the PDB structure 1XQ8 (See Figure 7) with the software PyMol. The proximity of Met127 to Tyr125 drew our attention, because we expected electron tunneling to occur between the two residues. We based our prediction on the theory of electron-transfer through proteins.

Electron-transfer from tyrosine to oxidized methionine has been reported in many studies. The mechanistic route for this electron-transfer occurs either via the formation of a two center-three electron bond, or via the formation of a bond between the sulfur of methionine and Br2 (Met/S⋅⋅⋅Br+) or via the formation of a bond between the sulfur radical cation of methionine and the electron pair of a nitrogen atom or an oxygen atom (Met/S⋅⋅⋅N+ or Met/S⋅⋅⋅O+) or via the formation of a bond between the sulfur radical cation of methionine and the sulfur of another methionine ((Met)2/S⋅⋅⋅S+). The species Met/S⋅⋅⋅N+ has an electrode potential $E^\circ = 1.4$ V at pH 6.8. Two studies reported experimental evidence of the formation of the Met/S⋅⋅⋅N+ and Met/S⋅⋅⋅O+ species upon oxidation of n-acetylmethionine amide and of
methionine in peptides\textsuperscript{11,12}. Nauser \textit{et al.} reported experimental evidence of Met/S::N\textsuperscript{+} formation with subsequent tyrosyl radical formation in the protein calmodulin\textsuperscript{16}. Mozziconacci \textit{et al.} presented experimental evidence of oxidized methionine repair through the oxidation of Tyr into a tyrosyl radical with the subsequent formation of dityrosine dimer in Met–enkephalin\textsuperscript{17}. Pulse radiolysis evidence of intramolecular addition of cysteine thiol radical to phenylalanine also hint at the possibility of interaction between the methionine sulfur radical cation and an aromatic residue\textsuperscript{18}. Finally, Monney \textit{et al.} used photoelectronspectroscopy to show that interaction with a \(\pi\)-sytem can stabilize a sulfur radical cation by the formation of a two center-three electron S::\(\pi\) bond\textsuperscript{19}.

We decided to use pulse radiolysis to subject synthetic peptides whose sequences reproduce the peptide environment of Tyr\textsubscript{125} and Met\textsubscript{127} of \(\alpha\)-synuclein to oxidative conditions. We recorded the formation of radical species by spectrophotometry. The C-terminal domain of \(\alpha\)-synuclein is unfolded in its native state, even when \(\alpha\)–synuclein interacts with a lipid membrane\textsuperscript{20}. This is a strong indication that our results obtained with synthetic peptides without secondary structure may be extrapolated to \(\alpha\)-synuclein.

We selected various sequences of interest that correspond to sequence parts of \(\alpha\)–synuclein and \(\beta\)–synuclein and performed experiments on the corresponding synthetic peptides. One sequence of interest is AYEMPSE, which corresponds to residues 124 to 130 in the \(\alpha\)-synuclein sequence, where we expect to observe an intramolecular electron transfer between oxidized methionine and tyrosine. We performed comparative studies between the synthetic peptide Ac-AYEMPSE-NH\textsubscript{2} (AE7) and the synthetic peptides Ac-AYAMPSE-NH\textsubscript{2} (AE7-E3A), Ac-AYEMASE-NH\textsubscript{2} (AE7-P5A), and Ac-AYPEAPSE-NH\textsubscript{2} (AE7–M4A) to investigate the influence of peptide sequence on intramolecular electron-transfer between oxidized methionine and tyrosine. Other sequences of interest include Ac-AYEMPSEEGYQDYE-NH\textsubscript{2} (AE14) and Ac-SYEDPPQEEYQEYE-NH\textsubscript{2} (SE14). The former sequence corresponds to residues 124 to 138 on the C-terminal domain of \(\alpha\)-synuclein and the latter sequence corresponds to residues 118 to 131 on the C-terminal domain of \(\beta\)-synuclein.

Our main finding is that there is an interaction in AE7 between tyrosine and methionine when they are subjected to oxidation. The intramolecular electron transfer between the tyrosine and the oxidized methionine is reversible, with the transfer from tyrosine to methionine radical being the
faster transfer. This finding is based on pulse radiolysis experiments with the hydroxyl radical as the initial oxidant of AE7 and AE7-M4A. The pH-dependence of the electrode potential of the AE7′/AE7 couple indicates that more than one electron is transferred at the electrode surface whereas AE7-M4A delivers just one electron per pH unit. We conclude that methionine in AE7 delivers the extra electron.

3.2 Results

3.2.1 Oxidation of Ac–AYEMPSE–NH₂ and intramolecular electron transfer

3.2.1.1 Comparison between the reaction of Ac–AYEMPSE–NH₂ with Ag²⁺ and the reaction of Ac–Tyr–NH₂ + Ac–Met–NH₂ with Ag²⁺

Ag²⁺ reacts with methionine 3.5 faster than it does with tyrosine²¹,²². We wanted to use Ag²⁺ to target methionine and observe whether oxidized methionine can oxidize a tyrosine in its vicinity. We oxidized AE7 with Ag²⁺ generated by pulse radiolysis. The solution contained 0.20 mM of AE7, 10 mM of AgNO₃, 2.0 mM HNO₃, and was saturated with N₂O before irradiation with a 50 ns pulse of 80 Gy (Fig. 1 & inset, curve a). The pH was 2.7. The electrode potential of Ag²⁺ is $E^\circ(\text{Ag}^{2+}/\text{Ag}^+) = 1.980 \text{ V}_2^{23}$, between that of HO⁺ ($E^\circ(\text{HO}^+, \text{H}^+/\text{H}_2\text{O}) = 2.73 \text{ V}_2^{24}$) and that of N₃⁻ ($E^\circ(\text{N}_3^-, \text{N}_3^-) = 1.33 \text{ V}_2^{25}$). Under these conditions we expected the following reactions, after irradiation (Reaction 1):

<table>
<thead>
<tr>
<th>Reactions</th>
<th>$k$ (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O ⇔ $e_{aq}^-(2.75)$, H⁺(0.55), HO⁺(2.75)</td>
<td>1</td>
</tr>
<tr>
<td>$e_{aq}^- + \text{N}_2\text{O} \rightarrow$ HO⁺ + N₂ + HO⁻</td>
<td>$8.9 \times 10^9$²⁶⁻³⁰</td>
</tr>
<tr>
<td>H₂O</td>
<td>2</td>
</tr>
</tbody>
</table>
The number of product molecules per 100 eV of deposited energy (G-value) of aqueous electrons (e_{aq}^{-}), hydroxyl radicals (HO'), and hydrogen atoms (H'), are G(e_{aq}^{-}) = 2.75, G(HO') = 2.75, and G(H') = 0.55\textsuperscript{71}, respectively. We worked at pH = 2.7; at higher pH we observed the formation of a direct reaction product between Ag\textsuperscript{+} and AE7 with the solution turning yellow. At low pH, however, reaction 2a competes with reaction 2. Based on the rate constants k\textsubscript{2}, k\textsubscript{2a}, k\textsubscript{3}, and k\textsubscript{4}, the G-value of Ag\textsuperscript{0} is (Ag\textsuperscript{0}) = 2.0. Based on the rate constants k\textsubscript{2}, k\textsubscript{2a}, k\textsubscript{3}, and k\textsubscript{6}, the G-value of Ag\textsuperscript{2+} is (Ag\textsuperscript{2+}) = 2.7. Given the high concentration of Ag\textsuperscript{+}, we expect that the very reducing Ag\textsuperscript{0} (E\textsubscript{o}(Ag\textsuperscript{+}/Ag\textsuperscript{0}) = -1.8 V)\textsuperscript{72} will react quantitatively with Ag\textsuperscript{+}, reaction 5. There are additional reactions, such as recombination of Ag\textsuperscript{2+} to form Ag\textsuperscript{4+} (Reaction 7).

An additional complication stems from the nitrate in the solution (Reactions 9 and 10). We expect the NO\textsubscript{2}\textsuperscript{−} formation with a G–value of G(NO\textsubscript{2}\textsuperscript{−}) = 0.42. Secondary reactions are known
and expected (Reaction 14). Given the applied doses, we calculated formation of 8 – 9 µM of Ag\(^{2+}\). We used the peptides in concentrations > 0.10 mM, and we are, therefore, safe in assuming that the Ag\(^{2+}\) oxidations follow pseudo-first-order kinetics (Reactions 12 and 13). The direct reactions of HO’ and H’ with the tyrosine and methionine in AE7 (Reactions 16, 17 and 18) have low yields, < 2%. Under our experimental conditions, the radical recombination reactions are slower than the (pseudo-) first order reactions of interest, because the concentration of radicals was very low. Therefore, we may safely assume that the initial radical – molecule reactions are

Figure 16: Formation and decay of Tyr/O’ upon oxidation by Ag\(^{2+}\). The conditions are the same for all curves: AgNO\(_3\) 10 mM, HNO\(_3\) 2 mM, pH 2.7 at room temperature. Solutions were saturated with N\(_2\)O. Detection at \(\lambda = 400\) nm. The average radiation dose of 26 Gy generated by 20 to 50 ns pulses of 2 MeV electrons. A; Formation of Tyr/O’ by reaction of 0.20 mM N-Ac-AYEMPSE-NH\(_2\) with 9 µM Ag\(^{2+}\). B; Formation of Tyr/O’ by reaction of 0.20 mM N-Ac-Tyr-NH\(_2\) and 0.20 mM N-Ac-Met-NH\(_2\) with 9 µM Ag\(^{2+}\). C; Formation of 8 µM Ag\(^{2+}\) by reaction of 10 mM AgNO\(_3\) with 9 µM HO’.
unaffected by recombination processes and that the highly complex silver chemistry did not affect our conclusions adversely.

We then compared the absorbance vs. time traces at $\lambda = 400$ nm of three solutions after irradiation: Ag$^+$ with AE7 (Figure 16 and inset, trace A), Ag$^+$ with Ac-Tyr-NH$_2$ and Ac-Met-NH$_2$ (Figure 16 and inset, trace B) and, as a control, a solution of Ag$^+$ (Figure 16 and inset, trace C). In all experiments, the peptide concentration was [peptide] = 0.20 mM, the silver nitrate concentration was [AgNO$_3$] = 10 mM, and the nitric acid concentration was [HNO$_3$] = 2 mM. The solutions were saturated with N$_2$O (24 mM). We expect Ag$^{2+}$ or Ag$_2^+$ species to form from reactions 5 and 6. Both species absorb at $\lambda = 400$ nm with comparable extinction coefficients$^{73,74}$, therefore, a part of the observed initial absorption at $\lambda = 400$ nm is due to both Ag$^{2+}$ and Ag$_2^+$ (Figure 16 traces A and B). The subsequent absorbance vs. time traces differ significantly between different solutions: with solution C, an immediate subsequent decay occurs. With solution B, absorption first increases slightly over 10 $\mu$s before it decreases. With solution A, absorption increases distinctly over 35 $\mu$s, and it then decays with a slower rate than that of solution B.

3.2.1.2 Reaction of Ac–AYEMPSE–NH$_2$ with HO$^-$

For the detection of the reaction of AcTyrNH$_2$ or AE7 with HO$^-$, we chose three wavelengths of particular interest: 295 nm, 340 nm and 405 nm (see below, Figure 17, Panels A, B, and C). We focused on the reactions of tyrosine and HO$^-$. At 340 nm, we monitored the adduct of the hydroxyl radical to tyrosine (Reaction 17). We observed additional phenomena farther in the UV. Because tyrosine strongly absorbs below 285 nm and therefore blocks the light transmission through our 6.0 cm cell, our lowest detection wavelength was 295 nm. Tyrosyl radicals exhibit a very sharp band at 405 nm that is commonly used for quantification (Reaction 27). It should be noted that radicals associated with the one–electron oxidation of methionine (reactions 18 – 25) exhibit distinct absorptions at all three wavelengths (see introduction). In AE7, the HO$^-$ radicals react primarily with tyrosine at a rate constant of $k_{17} = 1.3 \times 10^{10}$ M$^{-1}$ s$^{-1}$ $^{66-68}$ and methionine with a rate constant of $k_{18} = 8.1 \times 10^9$ M$^{-1}$ s$^{-1}$ $^{69,70}$. 
<table>
<thead>
<tr>
<th>Reaction</th>
<th>$k$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr/OH + HO$^+$ → Tyr/OH(−HO$^-$)</td>
<td>17</td>
</tr>
<tr>
<td>Met + HO$^+$ → Met/S−OH$^-$</td>
<td>18</td>
</tr>
<tr>
<td>Met + HO$^+$ → Met/HC$_γ^+$−S−CH$_3$</td>
<td>4.5 × 10$^9$ $^{75}$</td>
</tr>
<tr>
<td>Met + HO$^+$ → Met/CH$_2$−S−C$_ε$H$_2$</td>
<td>4.5 × 10$^9$ $^{75}$</td>
</tr>
<tr>
<td>Met/S−OH → Met/S$^+$ + HO$^−$</td>
<td>21</td>
</tr>
<tr>
<td>Met/S$^+$ → Met/HC$_γ^+$−S−CH$_3$ or Met/CH$_2$−S−C$_ε$H$_2$</td>
<td>2.5 × 10$^5$ $^{75}$</td>
</tr>
<tr>
<td>Met/S$^+$ → Met/S$^+$ : N$^+$</td>
<td>23</td>
</tr>
<tr>
<td>Met/S$^+$ → Met/S$^+$ : O$^+$</td>
<td>24</td>
</tr>
<tr>
<td>Met/S$^+$ + Met/S → Met$_3$/S$^+$ : S$^+$</td>
<td>25</td>
</tr>
<tr>
<td>Met$_2$/S$^+$ : S$^+$ + → 2 Met + Tyr/O$^+$</td>
<td>2.3 × 10$^7$ $^{10}$</td>
</tr>
<tr>
<td>Tyr/OH</td>
<td></td>
</tr>
<tr>
<td>Tyr/OH(−HO$^-$) + H$^+$ → Tyr/O$^+$ + H$_2$O</td>
<td>27</td>
</tr>
<tr>
<td>Tyr/OH + HO$^+$ → Tyr/O$^+$ + HO$^−$</td>
<td>28</td>
</tr>
</tbody>
</table>
Figure 17: Reaction of HO• with Ac–Tyr–NH₂ (Red) and Ac–AYEMPSE–NH₂ (Blue). Each graph was obtained from a solution with a peptide concentration of 0.1 mM in 5 mM phosphate, pH 7.4. Solutions were saturated with N₂O. A; λ = 295 nm. B; λ = 340 nm. C; λ = 405 nm.

At λ = 405 nm, we observed that the initial absorption in the presence of AE7 (Figure 17, Panel C, blue curve) is five times larger than in presence of Ac–Tyr–NH₂ (Figure 17, Panel C, red curve); the maximum absorption with AE7 is 30 % higher than that with Ac–Tyr–NH₂ (Figure 17, Panel C, inset). With Ac-Tyr-NH₂ (Figure 17, Panel B and C, Red curve), the rate of product formation at λ = 405 nm (k = 9.1 × 10⁴ s⁻¹) is equal to the decay rate at λ = 340 nm (k = 8.7 × 10⁴ s⁻¹). The two processes are likely linked: we observed formation of the tyrosyl radical (reaction...
27) coupled to the decay of the cyclohexadienyl radical (reaction 17). The maximum absorption at \( \lambda = 340 \) nm is 20 % higher in experiments with Ac–Tyr–NH\(_2\) than in those with AE7 (Figure 17, Panel B, inset); the absorption decay is also faster. At 295 nm, we found an identical initial absorbance for both peptides (Figure 17, Panel A). However, the decay kinetics at 295 nm and 340 nm are faster in the case of Ac–Tyr–NH\(_2\). The kinetics clearly change after approximately 200 \( \mu \)s, and the absorbance vs. time signal reaches a plateau (Figure 17, Panels A and B). Such a drastic change is not observed with AE7.

Earlier studies reported that proline stabilizes MetS\(^+\) better than other amino acids via reaction 23\(^{15}\). We compared AE7 with a mutant where proline is replaced by alanine, AE7–P5A. We were unable to find any difference in the kinetics of the two peptides (Figure 18A). There was also no difference in the kinetics between AE7 and the mutant AE7–E3A, where the glutamate next to the tyrosine was replaced by alanine (Figure 18B).
With Ag$^{2+}$ as the oxidant we observed that the yield of tyrosyl radicals is higher with AE7 compared to Ac-Tyr-NH$_2$ or a mixture of Ac-Tyr-NH$_2$ and Ac-Met-NH$_2$. An earlier paper reported that Tyr is oxidized intramolecularly by MetS$^{17}$+. As a control, we oxidized AE7 and its mutant Met4Ala AE7-M4A with HO’ (\textsuperscript{.}). The maximum absorption at 405 nm is higher in AE7 than in AE7-M4A (Panel B, inset). Likewise, the rate of the absorption increase is at least one order of magnitude faster in AE7 than in AE7-M4A (Panel B, inset). In the case of the mutant AE7-M4A, the absorption decrease at 350 nm and the increase at 405 nm are correlated. One should note that the decay of the tyrosyl radical in AE7 is clearly faster than in AE7-M4A.
3.2.3 Oxidation of Ac-AYEMSPPEEGYQDYE-NH₂: comparison with oxidation of Ac-SYEDPQPEEYQEYE-NH₂

3.2.3.1 The transient spectrum of the reaction of Ac-EAYEMSPPEEGYQDYEPEA-NH₂ and HO’ is the weighted sum of that of Ac-AYEMSPPE-NH₂ and HO’ and of Ac-EGYQDYE-NH₂ and HO’

The sequence AYEMPSE represents 5 % of the total α-synuclein sequence. Three of its four tyrosines are found in the C-terminal domain. In order to compare Tyr127 of α-synuclein, whose equivalent is found in AYEMPSE, to the two other tyrosine residues Tyr133 and Tyr136 in the C-terminal domain, we studied the reaction of HO’ with the synthetic peptide Ac-EAYEMSPPEEGYQDYEPEA-NH₂ (EA18), which corresponds to the sequence of amino acids 123 to 140 in α–synuclein.
We wanted to know specifically whether the presence of Met$_5$ close to Tyr$_3$ in EA18 made Tyr$_3$ more prone to oxidation than Tyr$_{11}$ and Tyr$_{14}$. To investigate, we opted to compare the spectrum of the reaction of EA18 and HO' to a spectrum that combined the spectra of the AE7 and HO' reaction and of the EE7 and HO' reaction. All spectra were obtained under the same conditions. We prepared solutions of peptide concentration of 0.20 mM in 100 mM phosphate buffers at neutral pH, all saturated with N$_2$O and analyzed the absorbance vs. time traces of these peptides reactions with HO'. We assumed that HO' would react with all residues other than methionine and tyrosine with equal rate constants. This approximation is acceptable because the rate constants of other amino acids and HO' reactions are all at least two orders of magnitude lower than the rate constants of Tyr and HO'; and Met and HO' reactions. Based on the rate constants published in the literature$^{66-69}$, we estimated that 44% of the HO' reacts with the AE7 and 56% with the EE7 if these two peptides were part of peptide EA18. We used these percentages as factors to weight the transient absorptions of AE7 and EE7 in order to combine their spectra.
Figure 20: Spectra of A; Ac–EAYEMPSEEGYQDYEPEA–NH₂, 0.2 mM (●). B; Ac–AYEMPSE–NH₂, 0.2 mM (●). C; Ac–EGYQDYE–NH₂, 0.2 mM (●). D; Comparison between the spectrum of Ac–EAYEMPSEEGYQDYEPEAq–NH₂ 0.2 mM (●) and the composite spectrum of Ac–AYEMPSE–NH₂ and Ac–EGYDYE–NH₂ (▲). Conditions: NaH₂PO₄ / Na₂HPO₄ 100 mM, pH 7.0, N₂O 24 mM.

The observations are the following. Whereas the formation of tyrosyl radical at λ = 400 nm has the same rate as the decay at λ = 300 – 360 nm in the case of EE7 (Figure 20 C, gradient–colored arrows), the formation of tyrosyl radical is faster than the decay at λ = 300 – 360 nm for both AE7 (Figure 20 B, blue and red arrows) and EA18 (Figure 20 A, blue and red arrows). As described above, the tyrosyl radical forms and decays faster in AE7 than a tyrosyl radical in a similar peptide without a methionine residue in its vicinity. There is no shoulder at λ = 320 nm in the case of AE7 (Figure 20 B, green arrow). Both EE7 (Figure 20 C, green arrow) and EA18 (Figure 20 A, green arrow), however, exhibit a shoulder at λ = 320 nm. The spectral changes associated with the radical in EA18 fit well to the weighed sum of AE7 and EE7 spectra in the range of 380 nm and above (Figure 20 D). There are significant differences in the region below 380 nm.

3.2.2.2 Comparison of the reaction of HO’ with Ac–AYEMPSEEGYQDYE–NH₂ (AE14) and Ac–SYEDPPQEEYQYE–NH₂ (SE14)

We intended to investigate whether there is a significant difference in the formation rates of tyrosyl radical between AE14 and SE14, due to the absence of a methionine residue in the latter. AE14 corresponds to residues 124 to 138 on the C-terminal domain of α–synuclein and SE14 corresponds to residues 118 to 131 on the C-terminal domain of β-synuclein. We prepared solutions of 0.20 mM of both peptides and exposed them to 25 µM of HO’. This ensured a pseudo-first-order reaction at pH 7.4. At an AE14 concentration of 0.20 mM, we assume that only Met/S⁺ forms because the formation of S::S⁺ is sterically hindered. This assumption is based on the observation that 0.50 mM of pentapeptide Met–enkephalin is low enough to prevent the formation of S::S⁺ species¹⁷. We measured the formation of products by spectrophotometry at two different wavelengths: λ = 360 nm and λ = 405 nm. In the case of SE14 (Figure 21, Panels C and D), the decay of the signal at 360 nm corresponds to the formation of the signal at 405 nm,
whereas in the case of AE14, (Figure 21, Panels A and B) the decay at 360 nm is slower than the increase at 405 nm by a factor of two.

Figure 21: Reaction of HO' with Ac-AYEMPSEEGYQDYE-NH₂ and Ac-SYEDPPQEEYQEYE-NH₂. Each graph was obtained from a solution with a peptide concentration of 0.20 mM in 5 mM phosphate, pH 7.4. Solutions were saturated with N₂O. A; Ac-AYEMPSEEGYQDYE-NH₂, λ = 360 nm. B; Ac-AYEMPSEEGYQDYE-NH₂, λ = 405 nm. C; Ac-SYEDPPQEEYQEYE-NH₂, λ = 360 nm. D; Ac-SYEDPPQEEYQEYE-NH₂, λ = 405 nm.

Another important observation is that decay of the signal in AE14 at λ = 405 nm is faster than that in SE14.
3.2.3 Voltammetry experiments with Ac-AYEMPSE-NH₂

3.2.3.1 Ac-AYEMPSE-NH₂ exchanges more than one electron per proton at the electrode surface

We decided to investigate the potential role of methionine on the oxidation of tyrosine by cyclic voltammetry. Comparisons made from observations of Figure 16, Figure 17, and Figure 19 show that the formation and decay of the tyrosyl radical in AE7 is promoted by the proximity of tyrosine to methionine. We wanted to test whether tyrosine and methionine influence their respective anodic currents. Influence by methionine on the anodic current that represents tyrosine oxidation would confirm the results presented in Figure 16, Figure 17, and Figure 19. According to the Nernst relation the ratio between the difference in electrode potentials and the difference in pH is 59 mV in the case of a 1e⁻/H⁺ electron transfer. In the case of AE7, if an electron transfers from methionine to oxidized tyrosine, we expect to see a deviation from this 59 mV value.

We compared the anodic currents of AE7 and AE7–M4A. After a first measurement with a freshly polished glassy carbon electrode, the anodic current of the voltammetric waves decreased stepwise after each further measurement until a steady–state was reached. We determined that this phenomenon is not due to coating of the electrode surface by peptides by comparing the shape of the experimental anodic wave with that predicted by the Matsuda equation. The Matsuda equation we used applies to the electrolytic current of a reversible redox reaction under linear sweep voltammetry conditions.

Given that the Matsuda equation for the reversible case fits the shape of the oxidation waves of AE7 and AE7-M4A, the reaction at the electrode surface is likely governed by diffusion, because we know that there is an equilibrium formed at the electrode surface and because the Matsuda equation is calculated with the hypothesis that the reaction is constrained by diffusion. Therefore, we can rule out adsorption as the cause of decreasing anodic current after each measurement. Another hypothesis is that loss of current occurs due to oxidative passivation of the carbon electrode by various carboxylic and alcohol species. We decided to analyze only the first voltammogram measured with a freshly polished glassy carbon electrode, because we do not entirely understand nor properly control this passivation process.
We observed that the oxidation wave starts at the same potential for all solutions at the various pH investigated, 7.2, 3.8, and 0.8; +0.51 V vs. Ag/AgCl (Figure 22, Panel A), +0.68 V vs. Ag/AgCl (Figure 22, Panel B), and +0.82 V vs. Ag/AgCl (Figure 22, Panel C), respectively. Beyond 1 V, the oxidation of water and the electrode surface started to contribute to the anodic current.

The difference between the maximum peak potential $E_p$ and the half–peak potential $E_{p1/2}$ value gives an indication of the reversibility of the reaction. When $E_p - E_{p1/2} = 57$ mV, the oxidation reaction at the electrode surface is reversible. The $E_p - E_{p1/2}$ value of AE7-M4A is the closest to 57 mV at all pH values. AE7 has $E_p - E_{p1/2}$ values significantly higher at pH 7.2 and pH 3.8, but $E_p - E_{p1/2}$ value is close to 57 mV at pH 0.8. These observations are also confirmed by how well the Matsuda equation fits the anodic wave currents measured. At pH 7.2 and 3.8, the Matsuda equation fits the anodic wave current of AE7 less well than it fits that of AE7-M4A, whereas it fits well with the anodic wave currents of both AE7 and AE7-M4A at pH 0.8 (Figure 22A, B, and C).
Figure 22: Linear sweep voltammograms of Ac–AYEMPSE–NH$_2$ 1mM, red symbol: $E_{p1/2}$, horizontal bar: standard error (■) and Ac–AYEAPSE–NH$_2$ 1.0 mM, red symbol: $E_{p1/2}$, horizontal bar: standard error (○), 2 mm glassy carbon electrode, 0.100 V/s. A; pH 0.8, B; pH 3.8, C; pH 7.2. Green curves: Fit of the Matsuda equation to the anodic wave current of Ac–AYEMPSE–NH$_2$. Blue curves: Fit of the Matsuda Equation to the anodic wave current of Ac–AYEAPSE–NH$_2$. 
Another observation is that half-peak potentials change order among the peptides across the investigated pH range. At pH 7.2, AE7 has the highest $E_{p1/2}$ (Figure 22 C, red square), whereas at pH 3.8 AE7 $E_{p1/2}$ is almost equal to AE7-M4A $E_{1/2}$ (Figure 22 B, red square and red circle, respectively). At pH 0.8, AE7 has the lowest $E_{p1/2}$ (Figure 22 A, red square).
The $E$ vs. pH diagrams of AE7 and AE7-M4A best illustrate this change in pecking order of the $E_{p1/2}$ (Figure 23). The slope of the $E$ vs. pH diagram of AE7-M4A is $59 \pm 1$ mV, whereas the slope of the $E$ vs. pH diagram of AE7 is $49 \pm 1$ mV.

### 3.2.3.2 Ac–AYEMPSE–NH$_2$ sequence does not influence the $pK_a$ of tyrosine

One explanation for the observation that tyrosyl radical can oxidize methionine is the following. The peptide environment might alter the $pK_a$ of the tyrosine, which in turn would influence its anodic current at different pH values. We measured the absorbance spectra of AE7 at pH 9.4 and pH 11.4 and compared them with the corresponding absorbance spectra of Ac-Tyr-NH$_2$ under the same concentration conditions. The hydroxyl group of tyrosine has a $pK_a$ of 10.4.$^{79}$
Compared to that of tyrosine, the UV–Vis spectrum of tyrosinate is shifted to the red. If the peptide sequence of AE7 influences the $pK_a$ of tyrosine, we would expect to observe different spectra between tyrosine and AE7 at either pH 9.4 or pH 11.4. The absorbance spectrum of AE7 is within the error that measured for Ac-Tyr-NH$_2$ (Figure 24). The interpretation that tyrosyl radical formation in AE7 is influenced by a $pK_a$ shift in tyrosine as a consequence of peptide environment can therefore be discarded.
Figure 24: Absorption spectra of Ac–Tyr–NH₂ 5 mM (Red) and Ac–AYEMPSE–NH₂ 5 mM (Blue). A; pH 9.4. B; pH 11.4
3.3 Discussion

3.3.1 Tyrosine can reduce the oxidized methionine residue in the peptide sequence Ac-AYEMPSE-NH$_2$ after reaction with Ag$^{2+}$

Figure 16 shows that the peptide sequence influences the reaction behavior of AE7 with Ag$^{2+}$. A yield ratio of about 25:75 of Tyr/O’-Met/S$^+$ could be reasonably expected upon reaction of AE7 with Ag$^{2+}$, because the reactions of Ag$^{2+}$ with phenol and methionine have rate constants of 1.0 $10^8$ M$^{-1}$ s$^{-1}$ 22 and 3.3 $\times$ $10^8$ M$^{-1}$ s$^{-1}$, respectively 21. At $\lambda$ = 400 nm, the absorbance vs. time signal of oxidized AE7 differs from that of the reaction of Ag$^{2+}$ with free Ac–Tyr–NH$_2$ and with Ac–Met–NH$_2$ reaction. We pointed out that the decay of tyrosyl radical in Ac–Tyr–NH$_2$ with Ac–Met–NH$_2$ and Ag$^{2+}$ (Figure 16, Trace B) is faster than in AE7 and Ag$^{2+}$ (Figure 16, Trace A). The possible explanations include dimerization of Tyr/O’; the reduction of Tyr/O’ by methionine; and the reduction of Tyr/O’ by Ag$^0$. Another side reaction that may occur is the recombination reaction of Ag$_4^{2+}$ with Tyr/O’ or Met$^+$. We will not further discuss these reactions here, because only the formation of tyrosyl radical is of interest in this discussion.

We can nonetheless make important interpretations from Figure 16 up to 100 µs. We observe the same fast initial absorption change in traces A, B, and C (Figure 16, inset, traces A, B, and C). In the case of the control, Ag$^{2+}$, (Figure 16, trace C) the initial absorption is followed by decay, and in the case of Ac-Tyr-NH$_2$ and Ac-Met-NH$_2$ with Ag$^{2+}$, (Figure 16, trace B) rapid product formation occurs over 10 µs before decay. However, in the case of AE7 with Ag$^{2+}$ (Figure 16, trace A), the fast initial product formation is followed by the slowest increase in absorption, with the largest absorption change. Only formation of a tyrosyl radical can explain the absorption increases observed in Ac–Tyr–NH$_2$ with Ac–Met–NH$_2$ and in AE7. The maximum absorption of oxidized AE7 at $\lambda$ = 400 nm is 70% higher than that of oxidized Ac-Tyr-NH$_2$ and Ac-Met-NH$_2$. We use the following two facts to provide the most plausible explanation of this observation. First, the major difference between AE7 and Ac-Tyr-NH$_2$ with Ac-Met-NH$_2$ is the constraint of the 15 Å distance between tyrosine and methionine in AE7. Second, Ag$^{2+}$ oxidizes methionine with a rate constant threefold faster than that of tyrosine and Ag$^{2+}$. Given these two facts, we interpret the yield of tyrosyl radical in AE7 as a consequence of
the initial formation of tyrosyl radical by direct reaction with Ag$_{2}^{+}$, followed by further tyrosyl radical formation via reduction of the oxidized methionine by tyrosine. The reason that we do not see the same yield and formation rate of tyrosyl radical in the mixture of Ac-Tyr-NH$_{2}$ and Ac-Met-NH$_{2}$ is the low concentration of Ac-Tyr-NH$_{2}$. The radical in Ac-Met-NH$_{2}$ cannot react fast enough with Ac-Tyr-NH$_{2}$ before it is reduced, either by dimerization or by Ag$^{0}$.

We bear in mind that we can only make a semi-quantitative interpretation because of the many complex interactions between the many compounds in solution. It is also worth noting here that silver cations may form complexes with the sulfur of methionine$^{80}$ and may also form complexes with a dityrosine dimer, as it forms complexes with catechol$^{81}$.

### 3.3.2 A reversible electron transfer occurs between tyrosine and methionine in Ac-AYEMPSE-NH$_{2}$

The comparison of the results obtained from AE7 with those obtained from Ac-Tyr-NH$_{2}$ (Figure 17) leads us to conclude that the absorbance vs. time traces of oxidized AE7 measured at $\lambda = 295$ nm, $\lambda = 340$ nm, and $\lambda = 405$ nm cannot be interpreted as consequences of the peptide methionine and the peptide tyrosine reactions with HO$^\cdot$. Based on the current literature we expect that tyrosine is oxidized by HO$^\cdot$ $^{66-68}$ and a secondary species formed after the oxidation of methionine such as Met/S$^\cdot$ $^{17}$, Met/S$^\cdot$.N$^\cdot$ or Met/S$^\cdot$.O$^\cdot$ $^{10}$.

We observed that the oxidized methionine in AE7 can be reduced by the tyrosine in its vicinity, but we observed no evidence for the formation of Met/S$^\cdot$.N or Met/S$^\cdot$.O. We discuss evidence based on the spectra we recorded and on the observation that oxidation of AE7, AE7-E3A, and AE7-P5A have the same kinetic behavior. Our set of experiments gives strong evidence for the direct oxidation of tyrosine by Met/S$^\cdot$, as reported earlier by Nauser and Schöneich$^{18,82}$ and by Mozziconacci et al.$^{17}$. We also report the reverse reaction, that is, reduction of the tyrosyl radical by methionine.

We excluded the possibility of formation of Met(S$^\cdot$.S)Met, which absorbs strongly from $\lambda = 350$ nm to $\lambda = 600$ nm$^{75}$, by conducting experiments at AE7 concentrations $\leq 0.20$ mM. We thus expected to see traces of different possible methionines mentioned above.
We can state confidently that there is neither a trace of Met/S:\=N, nor Met/S:\=O, nor of any other two center – three electron bond species in the absorbance vs. time traces we recorded. Beyond λ = 420 nm, there is insignificant absorption. Both Met/S:\=N, and Met/S:\=O absorb at this wavelength. Our observations do not match those made by Prütz et al. during their experiments of tyrosine oxidation by Met/S:\=Br+ in a L–Met – L–Tyr/OH peptide9. The absorption of oxidized AE7 after 7 µs and 70 µs at λ = 295 nm (Figure 17, Panel C) is higher than that at λ = 340 nm (Figure 17, Panel B) by a factor of 1.5 and by a factor of 3, respectively. The absorption of oxidized AE7 at λ = 405 nm is consistently the lowest over the full measurement time. At t = 7 µs, Prütz et al. observed that the trace at λ = 295 nm is lower than that at λ = 405 nm, whereas at t = 70 µs, it is higher. In summary, in the case of oxidized AE7, we observed that there is another pathway for the oxidation of tyrosine by oxidized methionine, which does not involve the formation of a two center-three electron bond.

The residues glutamate and proline next to methionine in AE7 could, in theory, provide electron density that allows the formation of either the Met/S:\=O+ or Met/S:\=N+ species. The observation that these residues had no influence on the kinetics the tyrosyl radical formation at λ = 405 nm (Figure 18) lends weight to our conclusion above.

It is, however, more difficult to assess the contribution of the species Met/S\=+, Met/C\γ\+, and Met/C\ε\+, to the absorbance vs. time traces that we recorded (Figure 17, Panel A). The absorption of Met/S\=+, Met/C\γ\+, and Met/C\ε\+, also occurs in the spectral region of m–Tyr–OH\+, o–Tyr–OH\+, and Tyr/O\+.

The most convincing evidence for the reversible electron transfer of tyrosine to oxidized methionine stems from the comparison between AE7 and AE7-M4A. The two peptides have the same length and same number of negatively charged residues. Therefore, we may assume that their steric and electrostatic effects are comparable. We observed that formation and decay of the tyrosyl radical is faster in AE7 than AE7-M4A when exposed to HO\+ (, Panel B). In AE7-M4A, the formation and decay of the tyrosyl radical are well separated, (, Panel B, black curve). The formation rate of the tyrosyl radical at λ = 405 nm also corresponds to the decay rate of the cyclohexadienyl radical at λ = 350 nm (, Panels A and B, black curve), like the formation of tyrosyl radical in the case of Ac-Tyr-NH2 (Figure 17, Panels B and C, red curve). This is not the case in AE7. There is no correspondence between the formation rate of the tyrosyl radical at λ =
405 nm and the decay rate of the cyclohexadienyl radical at $\lambda = 350$ nm (Panels A and B, blue curve). Moreover, in AE7, the decay of the tyrosyl radical overlaps with its formation (Panel B, blue curve). This difference between AE7 and AE7–M4A cannot be explained by differences in dimerization rates, because we may assume that the steric hindrances of AE7 and AE7–M4A are comparable, as explained above. Therefore, the only other alternative explanation is the presence of methionine in AE7. Methionine can reduce the tyrosyl radical next to it, and this would explain the faster rate of decay of the tyrosyl radical in AE7.

The voltammetry experiments support our interpretation that methionine can reduce the tyrosyl radical next to it in AE7. We observed noticeable differences in the $E$ vs. pH diagrams between AE7 and AE7–M4A. The $E$ vs. pH diagram of AE7–M4A has a slope value of $-dE^*/dpH = 59 \pm 1$ mV/pH (Figure 23) as would be expected for a 1 e$^-$ / 1 H$^+$ proton–coupled electron transfer. The $E$ vs. pH diagram of AE7, however, had a slope value $-dE^*/dpH = 49 \pm 1$ mV/pH (Figure 23). The value of the slope of the $E$ vs. pH diagram of AE7 deviates from the value expected for a 1e$^-$/1H$^+$ transfer. The extra electron transferred that causes this deviation must thus have come from the methionine. Methionine reduces the tyrosyl radical with one electron, and the reduced tyrosyl radical in turn transfers this electron to the electrode surface. Since re-protonation of the tyrosinate is 400 s$^{-1}$ at pH 7.2 and 10$^9$ s$^{-1}$ at pH 0.8, the second electron transfer is facilitated at higher pH, because it is not slowed down by re-protonation of the tyrosinate. We describe in the results section that the Matsuda equation fits the anodic current wave of AE7 less than it does that of AE7–M4A at pH 7.2 and 3.8. The secondary electron exchange with methionine may explain this observation. The electron transfer between methionine and oxidized tyrosine can couple to the reversible electron transfer from the electrode only to a limited extent if re-protonation of tyrosinate inhibits the electron transfer back to methionine (Figure 25).
Figure 25: Reversible electron transfer between oxidized tyrosine and methionine and competition between re-protonation of tyrosinate and reduction of oxidized methionine. Red frame: Scheme of reactions 31 and 32. Blue ellipse: Oxidation of tyrosinate at the surface of the electrode.

Two observations hint at the possibility that, to some extent, a second electron is transferred in the electrode reaction, provided by methionine over tyrosine. The first one is that the peak potential of the oxidation wave of methionine is $E_{p}(\text{Met}/\text{S}^{2+}/\text{Met}) \geq 1.3 \text{ V}$, and the half-wave potential that we measured at pH 7.2 for AE7, $E_{1/2p} = 0.71 \text{ V vs. Ag/AgCl}$, is much closer to the electrode potential of tyrosine, $E(\text{Tyr}/\text{O}^{+},\text{H}^{+}/\text{Tyr}/\text{OH}) = 0.97 \text{ V vs. NHE}$, No distinct second wave at higher potential was found. The second observation is that we measured a slope of 49 mV/pH from the AE7 $E$ vs. pH diagram, compared to the 59 mV/pH expected and found with the other peptides. If methionine directly transferred an electron to the electrode, a second wave should appear above 1 V vs. NHE, on top of the tyrosine wave. The slope of 49 mV/pH of the AE7 $E$ vs. pH diagram (Figure 23) derived for the tyrosine wave may therefore reflect electron transfer equilibrium between tyrosyl radical/methionine and tyrosinate/methionine radical cation, probably with bonding between the π system of tyrosinate and the radical cation. The dominant species in this equilibrium must be tyrosyl radical and methionine, otherwise the slope would be closer to 29.5 mV/pH.
The pulse radiolysis and voltammetry observations on AE7 support the following reactions:

\[
\text{Met/S}^+ + \text{Tyr/OH} \rightarrow \text{Met/S} + \text{Tyr/O}^+ + H^+ \quad (31)
\]

\[
\text{Met/S}^+ + \text{Tyr/OH} \rightarrow \text{Met/S} + \text{Tyr/O}^+ + H^+ \quad (32)
\]

The pulse radiolysis results presented in Figure 2 and Figure 5 established that reaction 33 does not occur:

\[
\text{Met/S} \cdot \cdot \cdot ^+ + \text{Tyr/OH} \rightarrow \text{Met} + \text{Tyr/O}^+ + H^+ \quad (33)
\]

Nauser et al. reported experimental evidence for reaction 33 in calmodulin\textsuperscript{16}. Whereas reaction 31 was already described in Met-enkephalin\textsuperscript{17}, we show here that Reaction 31 is reversible. This has not been reported before. Chen et al. suggested in a theoretical study that there is an electronic interaction between sulfur–centered radical cations and aromatic \(\pi\)-systems that lowers the ionization energy of methionine\textsuperscript{85}. The phenyl system can stabilize a sulfur–centered cation through an antibonding interaction between the \(p\)-type sulfur lone pair with the \(\pi\)-system to form a two center – three electron \(S:\pi\) bond\textsuperscript{85}. Monney et al. confirmed this suggestion when they provided experimental evidence by photoelectron spectroscopy\textsuperscript{19}.

We find that the proximity of methionine to tyrosine in AE7 leads to an electrochemical behavior different from that of a tyrosine without proximity to methionine. A similar statement can be made regarding experimental evidence we obtained by pulse radiolysis. We conclude that the proximity of methionine to tyrosine in AE7 increases both the formation and the decay rates of \(\text{TyrO}^+\) through a reversible electron transfer, as described in reactions 31 and 32, respectively.

We observed that the spectrum of EA18 fits the combined spectra of AE7 and EE7 (Figure 20). The differences observed at \(\lambda < 380\) nm may be due to folding effects that are caused by the length of EA18. The formation mechanism of tyrosyl radical in EE7 is similar to that of free tyrosyl radical. The formation and decay of the tyrosyl radical in EA18 share features with those of AE7 and EE7. We conclude that oxidants will attack all three tyrosine residues, Tyr125, Tyr133, and Tyr136, of \(\alpha\)–synuclein. The corresponding \(\beta\)–synuclein analog of AE14, SE14 (Figure 21, Panels C and D), behaves like Ac–Tyr–NH\(_2\) (Figure 17, Panels B and C, red curve).
3.3.3 Estimation of the electrode potential of Tyr:·Met/S⁺/TyrMet couple

We may picture the couple of reactions 31 and 32 as a two center – three electron bond between oxidized tyrosine and methionine, Tyr:·Met/S⁺.

Can we then measure the electrode potential of the couple of Tyr:·Met/S⁺? Our pulse radiolysis results do not allow us to measure the electrode potential of methionine in AE7, because we would need to determine the equilibrium constant \( K = \frac{[\text{MetS}^+]}{[\text{TyrO}^-]} \). As we explained above, we had to lower the optical resolution to increase the signal–to–noise ratio. As a result, the molar absorptivity of [Tyr/O⁺"] is not the “real” molar absorptivity. Furthermore, [MetS⁺"] cannot be measured accurately because it absorbs in the near–UV to violet region, where cyclohexadienyl radicals, as well as other carbon–centered radicals, absorb.

Our voltammetry results can provide us with an estimate of electrode potential of Tyr:·Met/S⁺. We base our estimate on an analogy of the Butler–Volmer equation:86

\[
j = j_0 \left\{ \exp \left[ \frac{\alpha_a n F (E - E^{°'})}{RT} \right] - \exp \left[ - \frac{\alpha_c n F (E - E^{°'})}{RT} \right] \right\}
\]

where \( j \) is the electrode current density in A/m², \( j_0 \) is the exchange current density in A/m², \( T \) is the absolute temperature in K, \( n \) is the number of electrons exchanged at the electrode surface, \( F \) is the Faraday constant in C/mol, \( R \) is the gas constant in J/(mol K), \( \alpha_c \) is the dimensionless cathodic transfer coefficient, \( \alpha_a \) is the dimensionless anodic transfer coefficient, \( E \) is the potential at the electrode in V, \( E^{°'} \) is the formal potential in V.

The Butler–Volmer equation applies to a reversible redox couple \( \text{Ox} + e^- \rightleftharpoons \text{Red} \). If \( \alpha_c = \alpha_a \), then \( k_t = k_r \). If \( \alpha_c > \alpha_a \), then \( k_t > k_r \). If \( \alpha_c < \alpha_a \), then \( k_t < k_r \). We use a Tyr-Met dipeptide that can transfer either one or two electrons to the electrode.

\[
\text{Tyr/OH–Met} \rightleftharpoons \text{Tyr/O}^-\text{–Met} + 1e^- + H^+ \quad E_{1^{°'}} \quad 34
\]
\[
\text{Tyr/OH–Met} \rightleftharpoons \text{Tyr/O}^-\text{–Met}^* + 2e^- + H^+ \quad E_{2^{°'}} \quad 35
\]

We assume that the reactions we investigate are reversible and that the two reactions are thermodynamically independent, yet kinetically coupled. We know that the first assumption is
fulfilled, because the Matsuda equation satisfactorily fits the voltammograms measured (Figure 22, green and blue curves). For the second assumption, we have no experimental evidence.

If the concentrations of the redox couples are at instantaneous equilibrium, then a $E$ vs. pH diagram would show the electrode potential as a linear function of pH with the standard electrode potential at pH = 0.

$$E^\circ = E^\circ' - \frac{EN}{n} \text{pH}$$  \hspace{1cm} (1)

Where

$$EN = RT/F \cdot 2.303 = 0.059 \text{ V at } T = 298 \text{ K}$$  \hspace{1cm} (2)

We know that $n$, the number of electrons exchanged at the electrode surface, is not a natural number. We will, therefore, replace $n$ by $\zeta$.

We then express the electrode potential of AE7 ($E_{AE7}^\circ'$) as:

$$E_{AE7}^\circ' = (1 - \alpha)E_1^\circ' + \alpha E_2^\circ'$$  \hspace{1cm} (3)

where $\alpha \in [0 - 1]$ and $\alpha$ represents the relative contributions of $E_1^\circ'$ and $E_2^\circ'$ to $E^\circ'$. Since the slope of equation (1) $-dE^\circ/d\text{pH} = EN/\nu$, we can also write equation (4):

$$\frac{EN}{\zeta} = \frac{EN}{1} (1 - \alpha) + \frac{EN}{2} \alpha$$  \hspace{1cm} (4)

By rearrangement of the terms in equation (4), we obtain:

$$\alpha = 2 - \frac{2}{\zeta}EN$$  \hspace{1cm} (5)

Since $\nu = -EN/(dE^\circ'/d\text{pH})$, equation (5) becomes:

$$\alpha = 2 - 2 \left(\frac{dE^\circ'/d\text{pH}}{EN}\right)$$  \hspace{1cm} (6)

We can then determine $\alpha$ from our results and replace it in equation (3). Based on our results, we calculate $\alpha$ to be 0.35. We take the electrode potential values $E^\circ'$ measured from the anodic waves recorded in AE7. For the $E_1^0$ values we take the effective electrode potential values measured from the anodic waves recorded in AE7–M4A. We chose AE7–M4A as a reference to
AE7 because AE7 and AE7–M4A have comparable electrostatic and steric factors. We can then estimate $E_2^\circ$ values.

Table 1: Values of $E_{an}^\circ$, $E_1^\circ$, and $E_2^\circ$, all corrected with the Nicholson equation

<table>
<thead>
<tr>
<th>pH</th>
<th>7.2</th>
<th>3.8</th>
<th>0.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{AE7}^\circ$ (V vs. NHE)</td>
<td>1.01</td>
<td>1.20</td>
<td>1.32</td>
</tr>
<tr>
<td>$E_1^\circ$ (V vs. NHE)</td>
<td>0.99</td>
<td>1.17</td>
<td>1.34</td>
</tr>
<tr>
<td>Estimated $E_2^\circ$ (V vs. NHE)</td>
<td>1.14</td>
<td>1.24</td>
<td>1.27</td>
</tr>
</tbody>
</table>

We mentioned above that we measured at a low scan rate, 0.1 V/s. At lower scan rates, there is competition between diffusion at the electrode surface, reduction reaction, and recombination of oxidized species. If recombination rate is high enough to offset the reduction rate, the concentration of oxidized product decreases fast enough to upset the reaction equilibrium. This results in lower measured electrode potentials. The most recent measurement of the electrode potential of tyrosine yields $E^\circ'(\text{Tyr/O}^\bullet, \text{H}^+/\text{Tyr/OH}) = 0.97$ V at pH 7. This measurement was performed at a scan rate of 400 V/s. Since we measured at a scan rate of 0.1 V/s, the difference factor is 4000. The Nicholson equation takes into account the influence of product recombination on the location of the peak potential:

$$E_p = E^\circ - \frac{RT}{3nF} \ln \left( \frac{4.78\pi nD_0}{3D_R} \right) - \frac{RT}{3nF} \ln \left( \frac{nFv}{k_c c_0 RT} \right)$$

Where $E_p$ is the peak potential, $R$ the gas constant, $T$ temperature in Kelvin, $n$ the number of electrons, here 1, $F$ the Faraday constant, $k$ the rate constant of the bimolecular recombination, $D_0$ and $D_R$ the diffusion coefficients of oxidized and reduced species, $c_0$ is the bulk concentration of the amino acid, and $v$ the scan rate. The equation predicts that the peak potential will be a function of the natural logarithm of the scan rate factorized by the ratio $RT/(3nF)$, where $R$ is the gas constant, $T$ is the temperature, $F$ is the Faraday constant, and $n$ is the number of electrons exchanged. At $T = 298$ K and $n = 1$, we can expect a difference in peak potential of 100 mV due to the difference factor of 4000.

These data allows us to calculate $E^\circ'(\text{Tyr/O'Met}^\bullet/\text{Tyr/O'Met})$ as 1.14 V vs. NHE at pH 7, if we take into account the correction to our measurements predicted by the Nicholson equation as
explained above. This value of 1.14 V vs. NHE at pH 7 may then also be the electrode potential $E^\circ'(\text{Met}^+/\text{Met})$ when methionine is located near a tyrosine. Figure 26 shows a Frost diagram that illustrates our findings. Given that the $E_2^\circ$ at pH 0.8 is lower than $E_1^\circ$, we calculate that TyrMet$^+$ can disproportionate to Tyr/OHMet and Tyr/O$^+$Met$^+$. The pH-dependence may indicate that protonation may have an effect on methionine oxidation in AE7. Our estimate of the two-electron contribution shows that the second electron transfer is not distinctly more difficult than the initial electron transfer from tyrosine.

The importance of electron transfer between oxidized methionine and nearby tyrosine in AE7 cannot be underestimated. Earlier and recent research on intramolecular electron transfer in proteins led to the conclusion that most oxidative damage eventually channels to the tyrosine residues, such as when tryptophanyl radicals oxidize tyrosine intramolecularly$^{7-9,87-92}$. The methionine residues are considered only as stepping stones to facilitate long–distance electron transfer$^{93}$.

Figure 26: Frost diagram with the electrode potentials of L-tyrosine and methionine next to tyrosine. YM: Tyr/OH–Met of equations 34-35. Met/S:C:N$: intramolecular two center-three electrons bond between sulfur and nitrogen in oxidized methionine. Blue line: slope = 1.14 V/n. Red line: slope = 0.99 V/n
We worked with carefully controlled anaerobic solutions in which the formation of the methionine sulfur radical cation can occur. As explained above, this sulfur radical cation can irreversibly form an (α-alkylthio) alkyl radical with the Cγ centred radical being the major radical. These carbon centred radicals could then react with O2 to form peroxyl radicals in aerobic conditions, because the rate constants for such reactions are \( k \approx 10^9 \text{ M}^{-1} \text{ s}^{-1} \) \(^{94,95} \). There is also the possibility homocysteine formation from oxidized methionine. To the best of our knowledge, such products have not yet been identified in α-synuclein subjected to oxidation conditions.

3.3.4 The rate constant of electron transfer from tyrosine to methionine sulfur radical cation lies within values as calculated with the Marcus-Levine equation

We measured the rate constant for formation of the tyrosyl radical from the tyrosine-hydridooxide(•) adduct (reaction 27) in AE7-M4A that is equal to \( k_{27} = 2.1 \times 10^4 \text{ s}^{-1} \). This value agrees with \( k = 1.8 \times 10^4 \text{ s}^{-1} \) measured by Solar et al. in 1984 \(^{68} \). This shows that the peptide sequence has no or little influence on the rate constant of reaction 27.

To calculate the rate constant for formation of the tyrosyl radical in AE7, we used a model of two parallel irreversible reactions that correspond to reactions 27 and 31. We calculated a rate constant for the reaction 31 of \( k_{31} = 3.2 \times 10^6 \text{ s}^{-1} \).

Gray and Winkler calculated that the distance decay constants for the coupling along β-strands and α-helices are \( \beta = 1.1 \text{ Å}^{-1} \) and \( \beta = 1.3 \text{ Å}^{-1} \), respectively. They also calculated the rate constants of electron transfer between donor and acceptor at a distance of 15 Å from one another of \( 2.0 \times 10^7 \text{ s}^{-1} \), \( 1.3 \times 10^6 \text{ s}^{-1} \), and \( 1.0 \times 10^5 \text{ s}^{-1} \) for coupling along β-strands, α–helices, and in water, respectively \(^{96} \). These values were calculated for Ru-azurin where \( \Delta G^\circ = -0.7 \text{ eV} \). The rate constant that they measured for a model protein Ruthenium-azurin is \( k = 6.0 \times 10^6 \text{ s}^{-1} \) \(^{96} \).

Because Tyr125 and Met127 in α-synuclein are in the disordered C-terminal end of α-synuclein\(^{20} \), we can assume that the distance decay constant for AE7 and α-synuclein lies between that of coupling along α-helices (β = 1.3 Å\(^{-1} \)) and that in water (β = 1.65 Å\(^{-1} \)). Therefore, we would expect the rate constant of electron transfer from Tyr127 to oxidized Met127 to lie between the values of \( k = 1.3 \times 10^6 \text{ s}^{-1} \) and \( k = 1.0 \times 10^5 \text{ s}^{-1} \).
We have to take into account that $\Delta G^\circ$ between tyrosine and methionine is different than that of the redox partners in Ru-azurin. We use the difference in Gibbs energy between $\text{Met}/S\cdot \text{N}^+$ and tyrosine $\Delta G^\circ = 1.42 \text{ eV}^{10} - 0.98 \text{ eV}^{84} = 0.44 \text{ eV}$, in our calculation of rate constant of electron transfer. Another difference to take into account is the value of the reorganization energy $\lambda$ for the peptide AE7 or the protein $\alpha$-synuclein. Gray and Winkler used a value of $\lambda = 0.7 \text{ eV}$ for Cu(II/I) in azurin. The value for electron self-exchange in $[\text{Cu(phen)}_2]^{2+/+}$ is $\lambda = 2.4 \text{ eV}^{97}$. We may reasonably assume that the reorganization energy value applicable to AE7 or $\alpha$-synuclein lies between these two values, because tyrosine and methionine in AE7 and $\alpha$-synuclein are more solvated than Cu(II) in azurin and are structurally less hindered than $[\text{Cu(phen)}_2]^{2+}$. For $\lambda = 0.7 \text{ eV}$ and $\Delta G^\circ = 0.44 \text{ eV}$, the rate constants are $k = 2.5 \times 10^6 \text{ s}^{-1}$ and $k = 1.9 \times 10^5$ for coupling along $\alpha$-helices and in water, respectively. For $\lambda = 2.4 \text{ eV}$ and $\Delta G^\circ = 0.44 \text{ eV}$, the rate constants are $k = 2.9 \times 10^3 \text{ s}^{-1}$ and $k = 2.2 \times 10^2$ for coupling along $\alpha$-helices and in water, respectively.

Our measurement of the formation rate constant for AE7 of $k_{31} = 3.2 \times 10^6 \text{ s}^{-1}$ is in the range as calculated by Gray and Winkler and correspond to a value expected at the top end for $\lambda = 0.7 \text{ eV}$ and $\Delta G^\circ = 0.44 \text{ eV}$, even though we would expect a higher distance decay constant for AE7 and $\alpha$-synuclein than that of coupling along $\alpha$-helices. Possible explanations to this observation is that distance between tyrosine and oxidized methionine is smaller than 15 Å or that the difference in standard Gibbs energy between tyrosine and methionine sulfur radical cation in its vicinity is lower than $\Delta G^\circ = 0.44 \text{ eV}$ (see section 3.3.3).

We may state that our measured rate constant for the electron transfer from tyrosine to oxidized methionine is close to the values measured for Ru-azurin proteins by Gray and Winkler and fits well with the theoretical framework provided by the Marcus-Levich equation (Section 1.4.2 equation 2).
3.4 References


(5) PyMOL The PyMOL Molecular Graphics System, V. S., LLC.; JyMOL The JyMOL Molecular Graphics System, V. S., LLC.; AxPyMOL The AxPyMOL Molecular Graphics Plugin for PowerPoint, V. S., LLC.


(83) Sanaullah; Wilson, G. S.; Glass, R. S. J Inorg Biochem 1994, 55, 87.
Chapter 4 \textit{\alpha–SYNUCLEIN OXIDATIVE DAMAGE AND ITS REPAIR BY MONOHYDROGEN ASCORBATE}

4.1 Introduction

Parkinson’s disease is a disease strongly linked to oxidative stress resulting from mitochondrial damage\textsuperscript{1-3}. This may explain the high concentration of ascorbic acid in neuron cells. Cortical neurons contain concentrations of ascorbic acid as high as 10 mM\textsuperscript{4,5}. These concentrations are about five folds higher than those of GSH (2-3 mM)\textsuperscript{5}. It is of interest to recall that ascorbic acid also performs other tasks in brain cells, such as sustaining neural maturation\textsuperscript{6}, facilitating neurotransmission\textsuperscript{7-12}, and partaking in collagen and myelin formation\textsuperscript{13} (see chapter 1). Ascorbic acid is central to the development of the brain, the viability of the fetus, and perinatal survival\textsuperscript{14-16}.

\textit{\alpha}–Synuclein is a protein recognized as being a target of oxidative stress, which results in the formation of stable \textit{\alpha}-synuclein dimers, possibly an onset to the formation of Lewy bodies\textsuperscript{17}. \textit{\alpha}-Synuclein exposed to oxidative stress forms nitrated oligomers stabilized by covalently cross-linked proteins via \textit{o,\textit{o}’}-dityrosine bonds\textsuperscript{17}.

A study established that Hasc\textsuperscript{−} reacts with tryptophanyl radical and with tyrosyl radical with a second-order rate constant ranging between orders of magnitude \textit{10\textsuperscript{7}} to \textit{10\textsuperscript{8}} M\textsuperscript{−1} s\textsuperscript{−1}\textsuperscript{18}. It was concluded that if Hasc\textsuperscript{−} can also react with other carbon centered radicals at such rates, then Hasc\textsuperscript{−} can neutralize protein radicals before nefarious damage to the cell occurs. We wanted to investigate whether Hasc\textsuperscript{−} can reduce tyrosyl radical in \textit{\alpha}-synuclein with a rate constant high enough to prevent the formation of \textit{o,\textit{o}’}-dityrosine.
4.2 Results

For the purpose of clarity in tables and in reactions, we abbreviated the tyrosyl radicals on AE7, AE14 and α-synuclein, with AE7/Tyr/O*, AE14/Tyr/O*, and SYNA/Tyr/O*, respectively.

We worked with peptide or protein samples of 1.0 mM or 2.0 mM concentration, that contained additionally 0.03 mM – 0.15 mM Hasc−, 0.10 M azide, and 5.0 mM phosphate buffer (pH 7.4). Solutions were saturated with N₂O. Azide was used in high concentration to ensure quantitative scavenging of HO’ (reaction 1). The equilibrium constant of reaction 2a is 0.3 M⁻¹ 19. At an azide concentration of 0.10 M, the concentration of hexanitride(•1–) (N₆⁻) is only 3 % that of trinitrogen(2N-N)(•) (N₃•).

Since the ascorbyl radical (asc•−) has its absorption maximum at 360 nm (ε₃₆₀ = 0.40 M⁻¹cm⁻¹) and the tyrosyl radical (Tyr/O*) at 405 nm (ε₄₀₅ = 0.32 M⁻¹cm⁻¹), we measured kinetics traces at these wavelengths. The dose was adjusted in such a way that the peptides or protein were 100-fold excess over N₃• and that the yield of reaction 2 was minimized. The calculated yield of tyrosyl radicals (reaction 3) is >90%. We observed a fast absorbance increase at λ = 405 nm, but the yield of tyrosyl radicals was below expectation: we calculated 56% for AE7, 85% yield for AE14, and 90% yield for α-synuclein.

<table>
<thead>
<tr>
<th>Reactions</th>
<th>k (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO’ + N₃• → HO⁻ + N₃•</td>
<td>1.2 × 10¹⁰</td>
</tr>
<tr>
<td>N₃⁺ + N₃⁺ → 3 N₂</td>
<td>4.5 × 10⁹</td>
</tr>
<tr>
<td>N₃⁺ + N₃⁻ → N₆⁺</td>
<td>2a</td>
</tr>
<tr>
<td>N₃⁺ + Ac–Tyr/OH–NH₂ → N₃⁻ + Ac–Tyr/O⁻–NH₂</td>
<td>1.3 × 10⁸</td>
</tr>
<tr>
<td>N₃⁺ + AE7/Tyr/OH → N₃⁻ + AE7/Tyr/O⁺</td>
<td>3a</td>
</tr>
<tr>
<td>N₃⁺ + AE14/Tyr/OH → N₃⁻ + AE14/Tyr/O⁺</td>
<td>3b</td>
</tr>
<tr>
<td>N₃⁺ + SYNA/Tyr/OH → N₃⁻ + SYNA/Tyr/O⁺</td>
<td>3c</td>
</tr>
<tr>
<td>N₃⁺ + Hasc⁻ → N₃⁻ + asc•− + H⁺</td>
<td>3.6 × 10⁹</td>
</tr>
<tr>
<td>2 Ac–Tyr/O⁻–NH₂ → Dimer</td>
<td>5</td>
</tr>
<tr>
<td>2 AE7/Tyr/O⁺ → Dimer</td>
<td>5a</td>
</tr>
<tr>
<td>2 AE14/Tyr/O⁺ → Dimer</td>
<td>5b</td>
</tr>
<tr>
<td>2 SYNA/Tyr/O⁺ → Dimer</td>
<td>5c</td>
</tr>
</tbody>
</table>
Given that \( k_4 = 30 \times k_3 \), there will always be some ascorbyl radicals formed via reaction 4 with the reactant concentrations we used. We give here an example with the lowest concentrations of Hasc\(^-\) and peptide that we used. At \([\text{Hasc}^-] = 0.03 \text{ mM}\) and \([\text{AE7}] = 2 \text{ mM}\), the expected ratio \([\text{asc}^-]:[\text{Tyr/O}^-] = 1:3\). Nevertheless, the measured absorption, \( G \times \varepsilon \), at \( \lambda = 405 \text{ nm} \) in AE7 is consistently 40% lower than the value derived from the published rate constants and extinction coefficients. In the case of the AE14 sample, the difference between the measured absorption and the value derived from the published rate constants is only 10% lower. In the presence of Hasc\(^-\), the decay of absorbance at \( \lambda = 405 \text{ nm} \) is accelerated proportionally to the concentration of Hasc\(^-\). AE14 has a decay rate (Table 2, row 2) roughly equal to that of AE7 (Table 2, row 1), and \( \alpha \)-synuclein (Table 2, row 3).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Rate constant (M(^{-1})s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE7</td>
<td>1.5 ( \pm ) 1.2 ( \times ) 10(^8)</td>
</tr>
<tr>
<td>AE14</td>
<td>1.5 ( \pm ) 2.8 ( \times ) 10(^8)</td>
</tr>
<tr>
<td>SYNA</td>
<td>1.9 ( \pm ) 5.2 ( \times ) 10(^7)</td>
</tr>
</tbody>
</table>
Figure 27: Kinetic traces at 360 nm and 405 nm. $k_{obs}$ vs [Hasc]. The conditions are the same for all graphs: 5.0 mM phosphate buffer, pH 7.4, 0.1 M N$_3^-$, 298 K. Solutions are saturated with N$_2$O. Dose: 5 Gy. Ascorbate concentrations: ◆: 0 mM / ●: 0.03 mM / □: 0.05 mM / ▲: 0.08 mM / ●: 0.10 mM / ★: 0.13 mM / *: 0.15 mM. Panel A: Ac-AYEMPSE-NH$_2$ 2 mM, 360 nm. Panel B: Ac-AYEMPSE-NH$_2$ 2.0 mM, 405 nm.
Panel C: Ac-AYEMPSEEGYQDYE-NH₂ 1.0 mM, 360 nm. Panel D: Ac-AYEMPSEEGYQDYE-NH₂ 1.0 mM, 405 nm. Panel E: α-synuclein 1.0 mM, 360 nm. Panel F: α-synuclein 1.0 mM, 405 nm.

Final absorption values of products at $\lambda = 405$ nm, as seen after $t = 800 \mu$s, are small in the case of AE7 for all Hasc– concentrations except for an ascorbate concentration of 0.20 mM. In AE7/Tyr/O’ and [Hasc–] = 0.03 mM, 0.05 mM, and 0.10 mM (Figure 27, panel B, ○, ■, □), and AE14/Tyr/O’ and [Hasc–] = 0.08 mM, 0.10 mM, and 0.13 mM (Figure 27, panel B, ▲, ●, ★), the absorption values as seen after $t = 800 \mu$s reach a plateau at about 0.5 mAU/Gy and 1.7 mAU/Gy, respectively. In AE7/Tyr/O’ and [Hasc–] = 0.20 mM, the final absorption values are 1.7 mAU/Gy (Figure 27 B, red ●).

Figure 28: A; The $k_{obs}$ for the decay of Ac-AY(Tyr/O’)EMPSE-NH₂ as a function of ascorbate concentration. B; The $k_{obs}$ for the decay of α-synuclein(Tyr/O’) as a function of ascorbate concentration.

In addition to these differences in final absorption values at $\lambda = 405$ nm, there were differences in kinetic behavior at $\lambda = 360$ nm between the samples. The initial absorption at $\lambda = 360$ nm is followed by a further increase in absorption with a rate matching that of the decay of the product at $\lambda = 405$ nm in the case of AE14, with 0.08 mM and 0.10 mM Hasc– (Figure 1, panel C&D, ▲, ●). The same observation is made for α-synuclein with 0.15 mM Hasc– (Figure 1, panels E&F, *). This observation is not made in the case of AE7. In AE7 with 0.05 mM and
0.10 mM Hasc\textsuperscript{−} (Figure 27, panel B, ■, ●), the initial absorption at \( \lambda = 360 \text{ nm} \) is followed almost immediately by a slow decay.

Figure 29: Comparison between the measured decay of tyrosyl radical in AE7 and the simulation obtained with the “Chemical Kinetics Simulator, version 1.01”. The conditions are: 5.0 mM phosphate buffer, pH 7.4, 0.1 M N\textsubscript{3}\textsuperscript{−}, 298 K. Solutions are saturated with N\textsubscript{2}O. A; ■: 2.0 mM Ac-AYEMPSE-NH\textsubscript{2} and 0.05 mM Hasc\textsuperscript{−}. Green curve: values obtained by the simulation. B; Green curve: residuals between the measurement and the simulation.

The disappearance of Tyr/O\textsuperscript{−} in AE7, reaction 5a, does not follow second–order kinetics (Figure 27, panel B, ●), as would be expected if only recombination of Tyr/O\textsuperscript{−} occurs. The Tyr/O\textsuperscript{−} in AE7/Tyr/O\textsuperscript{−} + Hasc\textsuperscript{−} = 0.03 mM, 0.05 mM, and 0.10 mM (Figure 27, panel B, ○, ■, ●) also doesn't decay at a first-order rate, as would be expected under the pseudo-first-order conditions that we set. We attempted to fit a reaction model to our data with the software “Chemical Kinetics Simulator, version 1.01” developed by IBM, but failed to do so. The green curve in Figure 29 A represents a simulation of reaction 3a in the presence of 0.05 mM Hasc\textsuperscript{−} (Figure 29 A, ■). We used the concentrations used during the measurements, the concentration of free
radicals expected on the basis of relevant published rate constants, and assumed that the decay of absorption is due to recombination of Tyr/O’ only. It is clear that, whereas the simulation fits our data at t = 0 µs and t = 900 µs, it fails to explain our data between t = 50 µs and t = 800 µs (Figure 29 B). The rates of the recombination of Tyr/O’ in AE14 and in α–synuclein, reactions 5b and 5c (Figure 27, panels D&F, respectively, ◆), are second-order as expected.

4.3 Discussion

4.3.1 N₃ is a suitable oxidant for our experimental design

N₃ is considered the reactant of choice to ensure the localization of the oxidative damage on tyrosine, since HO’ reacts unselectively not only at α-carbon sites on residues, but also with all major oxidation–prone residues such as cysteine, methionine, histidine, tryptophan, tyrosine and phenylalanine 27-30. N₃ reacts preferentially with tryptophan at a rate constant of $k = 4.8 \times 10^9 \text{M}^{-1}\text{s}^{-1}$ 22. α–Synuclein contains 1 histidine, 2 phenylalanine, 4 methionine, and 4 tyrosine residues 31. In the absence of tryptophan in α-synuclein, N₃ is the oxidant of choice for our experiments because it will react exclusively with the tyrosine residues in our peptides and protein of interest. We faced limitations because not only does N₃ react with itself 40× faster than it reacts with tyrosine, it also reacts with ascorbate 30× faster than it reacts with tyrosine. For meaningful experiments, the initial radical concentration has to be low, thus the dose was kept low. This results in lower signals and a lower signal-to-noise ratio. In order to decrease the noise level, the light intensity on the detector needed to be increased. Therefore, we had to lower the optical resolution, resulting in otherwise sharp spectral features, such as the absorption of the Tyr/O’ at 405 nm, appearing blurred and molar absorptivities appearing smaller than the real molar absorptivities. This makes it difficult to quantify the yields of the reactions that we studied. In our multi-pass cell, there is an additional issue with probes that scatter light. This was a problem with the protein samples, the transmission is lower than expected and we speculate that oligomers may be a reason for scattering. α-Synuclein also contains four tyrosine residues, therefore in a 6.0 cm cell a proper transmission can only be achieved with low α-synuclein concentrations such as 0.20 mM; a ten–fold lower concentration than that needed for meaningful
competition against ascorbate to scavenge N$_3^\cdot$. We can nonetheless make at least semi–quantitative evaluations of our data.

The yield of Tyr/O$^\cdot$ due to peptide/protein oxidation by N$_3^\cdot$ is as high as expected, $>90\%$, for AE14 (Figure 27, Panel D, ◆) and α–synuclein (Figure 27, Panel F, ◆). The Tyr/O$^\cdot$ yield in the case of AE7 (Figure 27, Panel B, ◆) is however much lower, slightly below 60%. This low yield cannot be explained by a dose that is too high, otherwise we would expect a Tyr/O$^\cdot$ yield as high as 70 to 80%. It is conceivable that the reaction between N$_3^\cdot$ and methionine is higher than the value of $k \leq 10^6$ M$^{-1}$s$^{-1}$ published by Land et al. $^{32}$ More likely, Tyr/O$^\cdot$ could be reduced by the sulfur of neighboring methionine, as suggested by the results we presented in chapter 3.

### 4.3.2 A process other than recombination occurs to explain the decay of tyrosyl radical (Tyr/O$^\cdot$) in AE7

Addition of ascorbate to the samples accelerates the absorption decrease at $\lambda = 405$ nm in all samples. The traces found did not allow for pseudo-first-order treatment of the reaction of AE7/Tyr/O$^\cdot$ with Hasc$^-$ (reaction 6a). It is possible that we used doses that were too high. We therefore attempted to simulate our results with the IBM software “Chemical Kinetics Simulator version 1.01”. We carried out the simulation under the assumption that the rate constant of the reaction of AE7/Tyr/O$^\cdot$ and Hasc$^-$ ($k_{\text{simulation}(6a)} = 1.4 \times 10^8$ M$^{-1}$s$^{-1}$) is slightly smaller than the rate constant $k(6a) = 1.5 \times 10^8$ M$^{-1}$s$^{-1}$ that we measured. The other assumption is that the rate constant of the recombination of AE7/Tyr/O$^\cdot$ is equal to the rate constant $k(5a) = 5.0 \times 10^2$ M$^{-1}$s$^{-1}$ that we measured (Figure 29 A, green curve).

Table 3: Parameters used for the model fit to reaction 6a

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{\text{simulation}(5a)}$</td>
<td>$5.0 \times 10^2$</td>
<td>M$^{-1}$s$^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{simulation}(6a)}$</td>
<td>$1.4 \times 10^8$</td>
<td>M$^{-1}$s$^{-1}$</td>
</tr>
<tr>
<td>$\varepsilon_{405}(\text{Tyr/O}^\cdot)$</td>
<td>$2.3 \times 10^3$</td>
<td>M$^{-1}$cm$^{-1}$</td>
</tr>
<tr>
<td>$\varepsilon_{405}(\text{asc}^\cdot)$</td>
<td>$2.0 \times 10^2$</td>
<td>M$^{-1}$cm$^{-1}$</td>
</tr>
</tbody>
</table>
We converted the concentrations of Tyr/O’ and asc⁻ to absorbance units using the extinction coefficients at \( \lambda = 405 \) nm for ascorbyl and Tyr/O’ listed in Table 3. We know from Bielski et al. that \( \varepsilon_{405}(\text{asc}^-) \) is at least smaller than 500 M\(^{-1}\)cm\(^{-1}\) \(26\). As we explained above, the molar absorptivity of the Tyr/O’ at \( \lambda = 405 \) nm that we measured is lower than the real molar absorptivity. Therefore, we assumed that the molar absorptivity that we measured is close to the lowest value of the extinction coefficient of Tyr/O’ at \( \lambda = 405 \) nm, \( \varepsilon_{405} = 2600 \) M\(^{-1}\)cm\(^{-1}\), published by Bensasson et al. \(33\). The subsequent fit obtained was not fully satisfactory and showed small systematic deviations (Figure 29 A, green curve and ■). Our current knowledge of the reaction mechanism in AE7 with \( \text{N}_3^- \) is too limited to provide a satisfactory explanation. We speculate that such deviations may originate in the reduction of the Tyr/O’ by methionine in AE7 (See chapter 3).

4.3.3 Monohydrogen ascorbate (Hasc⁻) can repair tyrosyl radicals (Tyr/O’) on \( \alpha \)-synuclein and prevent further oxidative damage

The reaction rate for the reaction AE7/Tyr/O’ and Hasc⁻ (reaction 6a) is \( k(6a) = 1.5 \times 10^8 \) M\(^{-1}\)s\(^{-1}\) (Table 2, first row / Figure 28 A). The reaction rate for the reaction AE14/Tyr/O’ and Hasc⁻ (reaction 6b) is \( k(6b) = 1.5 \times 10^8 \) M\(^{-1}\)s\(^{-1}\) (Table 2, second row / Graph not shown). The rate constant for the reaction SYNA/Tyr/O’ + Hasc⁻ (reaction 6c) is \( k(6c) = 1.9 \times 10^7 \) M\(^{-1}\)s\(^{-1}\) (Table 2, third row / Figure 28 B). A first–order reaction can be fitted well to the absorbance vs. time traces of the reactions 6b and 6c. The absorbance vs. time traces of the reaction 6a cannot fit a first–order reaction simulation. As a result, the rate constant that we reported in Table 2 for the reaction between AE7/Tyr/O’ and Hasc⁻ may be erroneous, because it appears that we would need to take into account unforeseen side reactions. Moreover, in AE14, the standard errors on the measured \( k_{\text{obs}} \) were the highest of all the samples (data not shown), and the ordinate of the \( k_{\text{obs}} \) as a function of ascorbate concentration was also very high, which suggests a high experimental error.

These three rate constants are to be compared with the rate constants of the reduction of Tyr/O’ in various proteins by Hasc⁻ published by Domazou et al. \(18\). The reduction of Tyr/O’ by Hasc⁻ of insulin, chymotrypsin, pepsin, lysozyme, and \( \beta \)-lactoglobulin are \( 2.9 \times 10^7 \) M\(^{-1}\)s\(^{-1}\), \( 4.0 \times 10^7 \) M\(^{-1}\)s\(^{-1}\), \( 1.9 \times 10^7 \) M\(^{-1}\)s\(^{-1}\), \( 6.5 \times 10^6 \) M\(^{-1}\)s\(^{-1}\), and \( 2.9 \times 10^6 \) M\(^{-1}\)s\(^{-1}\), respectively.
1s^{-1}, 3.5 \times 10^7 \text{M}^{-1}s^{-1}, 1.1 \times 10^7 \text{M}^{-1}s^{-1}, \text{and} 4.0 \times 10^5 \text{M}^{-1}s^{-1}, \text{respectively. In the case of} \beta\text{-lactoglobulin, it was established that the small rate constant in comparison to other proteins is due to steric hindrance and not due to a coulombic barrier}^{18}. \text{The rate constants that we measured are at the higher end of the rate constants reported by Domazou et al. This indicates that SYNA/Tyr/O}^• \text{is easily accessible by Has}^-\text{. The ascorbate concentration in neurons has been estimated to be as high as 10 mM (2 mM in glial cells)}^{4,5}. \text{If we take 2 mM as the basis for our calculation, the repair process of Tyr/O}^• \text{on } \alpha\text{-synuclein has a half–life time of 25 }\mu\text{s. Such a low half-life suggests efficient protection from further damage such as the formation of peroxyl Tyr/O}^• \text{or dimerization of SYNA. Our estimate of the second order of SYNA/Tyr/O}^• \text{recombination is } 1.7 \pm 0.8 \times 10^3 \text{M}^{-1}s^{-1}. \text{The concentration of tyrosyl radical in } \alpha\text{-synuclein necessary to obtain a half-life of 25 }\mu\text{s is 11 M. In this case, the ratio of tyrosyl radicals that undergo repair or dimerization is 50:50. If the ratio is to be 99:1, the concentration of tyrosyl radical still needs to be 200 mM. Such a high concentration of tyrosyl radical in } \alpha\text{–synuclein at any point in time is extremely unlikely, and we can confidently state that the reduction of tyrosyl radical in } \alpha\text{-synuclein by 2 mM monohydrogen ascorbate excludes the possibility of dimerization of tyrosyl radical in } \alpha\text{-synuclein.}

Hasc^- \text{can indeed play an important role in controlling the formation of Tyr/O}^• \text{in } \alpha\text{-synuclein, in addition to its other functions, such as sustaining neural maturation}^6, \text{facilitating neurotransmission}^{7-12}, \text{and partaking in the formation of collagen and myelin}^{13}.\n
4.3.4 Does a carbon centered radical in Ac-AYEMPSE-NH₂ react with ascorbyl radical?

Our results also indicate that the decay at } \lambda = 405 \text{ nm does not fit a purely second-order decay function in the AE7 sample, yet the experimental kinetics traces of AE14 and } \alpha\text{-synuclein fit a second–order decay function quite well, as we explained in the results section. Moreover, the ascorbyl radical yields are smaller in the AE7 sample than in the AE14 and } \alpha\text{-synuclein samples.} \text{Tyr/O}^• \text{in AE7 reacts faster with Has}^-\text{ than Tyr/O}^• \text{in } \alpha\text{-synuclein. However, in AE7 and with } [\text{Has}^-] = 0.03 \text{ mM, 0.05 mM, and 0.10 mM, the final absorption at } t > 800 \mu\text{s at } \lambda = 405 \text{ nm (Figure 27 B, } \bigcirc, \blacklozenge, \blacklozenge\text{, respectively) is lower than that in AE14 and } [\text{Has}^-] = 0.08 \text{ mM,
0.10 mM, and 0.13 mM (Figure 27 D, ▲, ●, ★, respectively). We expect an initial yield of 70% ascorbyl radical by direct reaction with N₃⁻ in AE7/Tyr/O’ and 0.20 mM of Hasc⁻. In AE7/Tyr/O’ and 0.20 mM Hasc⁻, the end absorbance at λ = 405 nm is 1.7 mAU/Gy (Figure 27 B, red ○). The final absorbance of 1.7 mAU/Gy likely represents only ascorbyl radical formed immediately by reaction 4. In contrast, in AE7/Tyr/O’ and 0.10 mM Hasc⁻ (Figure 27 B, ●), the end absorbance at λ = 405 nm is 0.5 mAU/Gy, a value to which other final absorption values at λ = 405 nm in solutions with 0.03 mM and 0.05 mM tend toward as well (Figure 27, Panel B, ○ and ■, respectively). With an ascorbate concentration of 0.10 mM, we can expect a half-life of 100 µs for Tyr/O’ in AE7, and by 800 µs Tyr/O’ should be quantitatively reduced. Therefore, only ascorbyl radicals would still absorb at t > 800 µs at λ = 405 nm with ε₄₀₅ = 5.0 × 10² – 7.5 × 10² M⁻¹ cm⁻¹. In AE14 and Hasc⁻, the absorbance at t = 900 µs at λ = 405 nm is 4 times higher than that in the sample AE7 and Hasc⁻ (Figure 27, C). The latter observation, along with the observation that the yield of AE7/Tyr/O’ is lower from the reaction between AE7 and N₃⁻, hints at the possibility that at least one other side reaction takes place in the reaction between AE7 and N₃⁻. This may be explained by studying the reaction of AE7 and HO’ in chapter 3. We found that methionine in AE7 can reduce the neighboring Tyr/O’. Gebicki and Nauser observed that ascorbyl radical can reduce carbon centered radical in GSH with dehydroascorbate as the end product. Given this observation and given that methionine sulfur radical cation dissociates readily into (α–alkylthio) alkyl radicals and a proton, we propose that the lower final absorption values in AE7, as seen after t = 800 µs, are due to the reaction between a carbon centered radical on methionine and an ascorbyl radical (Figure 30). This observation fits with our previous results about the formation of methionine radical when methionine is in the vicinity of Tyr/O’.
We chose to monitor the reactions at $\lambda = 405$ nm and $\lambda = 360$ nm to monitor Tyr/O$^\bullet$ and asc$^\bullet$, respectively. There is unavoidable competition between reactions 3 and 4 due to the high rate of reaction between Hasc$^-$ and N$_3^\bullet$. Hasc$^-$ can compete efficiently with tyrosine to scavenge N$_3^\bullet$, hence the initial absorption at $\lambda = 360$ nm. The correlation between the rate of formation of ascorbyl radical at $\lambda = 360$ nm and the rate of decay of tyrosyl radical at $\lambda = 405$ nm is also different between AE7 and AE14. In AE14, the reaction of AE14/Tyr/O$^\bullet$ with 0.13 mM leads to an immediate product formation at both $\lambda = 360$ nm and $\lambda = 405$ nm (Fig. 1, Panels C&D, ★). We conclude that both tyrosyl (30%) and ascorbyl radicals (66%) are formed simultaneously, as expected from the published rate constants for reactions 3b and 4. In AE14 with 0.08 mM and 0.10 mM, the absorbance at $\lambda = 360$ nm, as seen after $t = 10 \mu$s, forms at a rate comparable to the decay of the absorbance at $\lambda = 405$ nm (Figure 27, Panels C&D, ▲, ●). This would be expected
for the reduction of tyrosyl radical by Hasc−. Whereas the same observation is true in the case of α-synuclein with 0.15 mM Hasc−, this observation does not hold in the case of AE7 with 0.05 mM or with 0.10 mM. In AE7, there is a pseudo-first-order decay at \( \lambda = 405 \) nm proportional to the amount of Hasc− present, at \( \lambda = 360 \) nm, there is the immediate formation of product (Figure 27, Panels A&B, □, ●). The absorption at \( \lambda = 360 \) nm barely decays. It is possible that there is an isosbestic point at \( \lambda = 360 \) nm, where a product decays and another one forms at the same rate. It is likely that a quasi equilibrium exists between the formation and decay of ascorbyl radical due to the reduction of tyrosyl radical by Hasc− and the reduction of (α-thioalkyl) alkyl radical (See Chapter 3) by ascorbyl radical, respectively.

It is impossible to assess for sure whether the kinetics of the reaction AE7/Tyr/O’ + Hasc− (reaction 3a) at \( \lambda = 405 \) nm is of parallel first-order and second-order or is biexponential, that is, two parallel first-order reactions. In light of the results obtained in chapter three, it is plausible that Hasc− cannot scavenge all AE7/Tyr/O’ before it is reduced by neighboring methionine. If we take AE7/Tyr/O’ and 0.10 mM Hasc− as a reference, the reduction of Tyr/O’ by neighboring methionine would have a rate of at least \( k = 1.5 \times 10^4 \) s\(^{-1}\).
4.4 References


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Parkinson’s disease is linked to mitochondrial complex I deficiency and subsequent cellular oxidative stress conditions. α-Synuclein is a protein central to the disease etiology, chiefly due to its propensity to form fibrils called Lewy bodies; a hallmark of Parkinson’s disease. An interesting comparison is that of α-synuclein and β-synuclein. Although the two proteins are classified in the same protein family and are 59% homologous, the protein β-synuclein has a lower propensity to form fibrils.

The nitrated oligomers of α-synuclein that act as the initial seeds for fibril formation are stabilized by the formation of dityrosine bonds. α-Synuclein sequence at the C-terminal domain has a cluster of three tyrosine residues. Met127 is 15 Å away from Tyr125.

Two studies reported experimental evidence of the reduction of oxidized methionine by neighboring tyrosine. Nauser et al. reported experimental evidence of Met/S⁺·N⁺ formation with subsequent tyrosyl radical formation in the protein calmodulin. Mozziconacci et al. presented experimental evidence of oxidized methionine repair through the oxidation of Tyr into a tyrosyl radical with the subsequent formation of dityrosine dimer in Met–encephalin.

Domazou et al. established that monohydrogen ascorbate reacts with tryptophanyl radical and with tyrosyl radical with a second-order rate constant ranging between the orders of magnitude 10⁷ to 10⁸ M⁻¹s⁻¹. This makes monohydrogen ascorbate a suitable agent to reduce tyrosyl radicals in α-synuclein before it dimerizes. Cortical neurons contain concentrations of ascorbic acid as high as 10 mM.

We established in this study that there is a reversible electron transfer between Tyr125 and oxidized Met127 in α-synuclein. From our spectroscopic evidence, we established that no two center-three electron bond forms as an intermediate for the oxidation of Tyr125 by Met127, as was observed in other studies. Therefore, we conclude that the electron is exchanged on the sulfur atom of methionine.

A fraction of the sulfur radical cations that take part in this reversible electron transfer will dissociate into (α-thioalkyl) alkyl radicals and protons. This dissociation is irreversible, because Cε centered radical has a calculated pKₐ of -2.0 and the Cγ centered radical has a calculated pKₐ of...
of -6.0\textsuperscript{18}. These carbon centered radicals may lead to the formation of homocysteine, or may react with oxygen to form peroxyl radicals. The fate of these peroxyl radicals is poorly defined, and products of peroxyl radicals on other aliphatic amino acids lead to the formation of unstable hydroperoxides, alcohols, and carbonyl compounds\textsuperscript{19}. However, the only products identified from oxidation of methionine remain sulfoxide and sulfone\textsuperscript{19}.

We measured a second-order rate constant for the reaction of tyrosyl radical in \(\alpha\)-synuclein and monohydrogen ascorbate that is fast enough to prevent the dimerization of tyrosyl radical in \(\alpha\)-synuclein. We also observed in Ac-AYEMSPE-NH\(_2\) that monohydrogen ascorbate cannot react with tyrosyl radical fast enough to prevent the formation of sulfur radical cation in methionine and the subsequent formation of (\(\alpha\)-thioalkyl) alkyl radical. This observation is of interest, and it would be interesting to conduct a control experiment to investigate the reaction of tyrosyl radical in Ac-AYEAPSE-NH\(_2\), a peptide that lacks methionine in the vicinity of tyrosine, and monohydrogen ascorbate. If our interpretation of our observation is correct, the ascorbyl radical yield in Ac-AYEAPSE-NH\(_2\) should be higher than that in Ac-AYEMPSE-NH\(_2\).

If we are to extrapolate our interpretation of the high reaction rate of tyrosyl radical in \(\alpha\)-synuclein and monohydrogen ascorbate to the intracellular neuron environment, we have to evaluate a key assumption. As explained in the introduction, stable oligomers with \(o,o'\)-dityrosine cross-links are nitrated \textit{in vivo}\textsuperscript{3}. We conducted our experiments with trinitrogen(2\(N\)-\(N\))(\(\bullet\)) as the initial oxidant. Does tyrosyl radical in \(\alpha\)-synuclein formed by the reaction with trinitrogen(2\(N\)-\(N\))(\(\bullet\)) form stable oligomers that then lead to the formation of fibrils? If it were the case, then the validity of our prediction, namely that monohydrogen ascorbate can prevent the formation of stable \(\alpha\)-synuclein oligomers, would be supported by strong evidence.

The other question that remains is whether the low electrode potential of Met127 due to its vicinity to Tyr125 and the reversible electron transfer from Tyr125 to oxidized Met127 contribute to the formation of stable \(\alpha\)-synuclein oligomers. Oxidized dopamine can interact directly with \(\alpha\)-synuclein and induce the oligomerization of the latter\textsuperscript{20}. The molecular effects of dopamine toxicity were also investigated. It was established that dopamine metabolism is associated with \(\alpha\)-synuclein related cytotoxicity. Neurons were transformed with one of the four following mutants of \(\alpha\)-synuclein, Tyr125Asp, Met127Ala, Met116Ala/Met127Ala, or Ser129Ala, with all of these mutants exhibiting lower levels of methionine sulfoxide\textsuperscript{21}. 

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α-Synuclein has four methionine residues, but only Met116 and Met127 are modified under conditions of oxidative stress. We cannot link our results to this observation, because we investigated a single-electron oxidation reaction, and because the intracellular environment of neurons is more complex than the solutions we used for our experiments. The correlation is, however, of interest. β-synuclein lacks a residue equivalent to Met127 in α-synuclein, but it has a residue equivalent to Met116. Is this difference part of the explanation as to why β-synuclein is less prone to oligomerization than α-synuclein? In comparison to α-synuclein, β-synuclein has a similar NAC-domain, except for the lack of the sixth imperfect repeat of KTKEGV, and tyrosine and methionine residues equivalent to the residues in α-synuclein, and except for the absence of a methionine residue equivalent to Met127 in α-synuclein. We established that both tyrosyl radical formation and decay in α-synuclein are faster than those in β-synuclein. It is a faint hint that the vicinity of Met127 to Tyr125 in α-synuclein may play a role in oligomer formation.
5.1 References


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