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Amide Neighbouring Group Effects in Peptides: Phenylalanine as Relay Amino Acid in Long-Distance Electron Transfer

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Abstract: Nature uses proteins as medium for long-distance electron transfer (ET) to carry out redox reactions in distant compartments. This ET occurs either by a single-step superexchange or through a multi-step charge hopping process, which uses side chains of amino acids as stepping stones. In this study we demonstrate that Phe can act as a relay amino acid for long-distance electron hole transfer through peptides. The considerably increased susceptibility of the aromatic ring to oxidation is caused by the lone pairs of neighbouring amide carbonyl groups, which stabilize the Phe radical cation. This neighbouring amide group effect helps to better understand the mechanism of extracellular electron transfer through conductive protein filaments (pili) of anaerobic bacteria during mineral respiration.

Electron transfer (ET) over long distances is a fundamental reaction in living organisms,[1] and in recent years extracellular electron transfer (EET) during microbial mineral respiration became a hot research topic.[2] During this process electrons migrate from their generation site at the inner cell membrane to metal ions outside of the cell, which are the final oxidants (Figure 1). Typical anaerobic bacteria capable of EET are of the *Geobacter* and *Shewanella* families.[2,3] These bacteria are used nowadays for the remediation of water, for example to remove toxic heavy metal and radionuclide contaminants such as Cr(VI), U(VI) or Tc(VII),[4] as well as for the development of microbial fuel cells,[5] and the synthesis of nanoparticles.[6] Aggregates of small proteins (pili) can function as medium for EET, and Lovley has observed that these protein filaments transfer electrons even in the absence of iron-containing cofactors (Figure 1a).[7] Thus, ET over several hundred of Å occurs through metal-free proteins, which raises the question of the mechanism by which such EET can take place. It was recently suggested that ET through proteins and peptides over 20 Å requires a multistep reaction,[8] which uses the side chains of relay amino acids as stepping stones. These stepping stones are reversibly oxidized to radical or radical cation intermediates, which break up one long ET step into several short ones (Figure 1b). This transition from a superexchange process to a hopping mechanism increases ET rates dramatically.[9] However, in order to function as relay stations for oxidative ET, amino acids should be readily oxidizable. Tyr, Trp, and Cys have redox potentials of around 1 V vs. NHE, and kinetic as well as spectroscopic experiments have demonstrated the formation of short lived intermediates during ET under oxidative conditions.[1f,8,10]

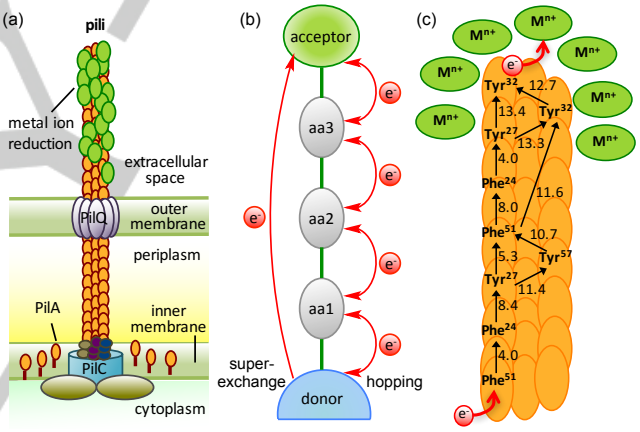

Figure 1. a) Extracellular electron transfer (EET) from respiration sites at the inner cell membrane to metal ions outside the cell during mineral respiration uses conductive protein filaments (pili). b) ET through peptides can proceed through a coherent tunnelling process (superexchange) or through a hopping mechanism using side chains of amino acids (aa1-3) as stepping stones. c) Pili transport electrons using Tyr and Phe as relay amino acids. Orange "footballs" are peptides that aggregate to the pili. Interaromatic distances in Å for the Asp2 pilus (cell constituents are omitted for clarity).[12]

Recently, Lovley,[11] and Reguera[12] proposed that Phe might also act as a relay amino acid, because the structural evaluation of pili revealed several Phe residues located at positions appropriate for ET (Figure 1c). Since the redox potential of alkylated phenyl groups is about 2 V vs. NHE,[13] this suggestion is astounding and raises the question whether Phe is more easily oxidizable in a peptide environment. For instance, Glass and Schöneich showed in recent pulse radiolysis experiments that the redox potential of dialkyl thioethers can be reduced by an adjacent amide group by over 0.5 V, which could transform Met into a relay amino acid.[14]

In order to determine the influence of amino acids on the ET rate and mechanism, we had developed assay **1** (Scheme 1).[10,15]

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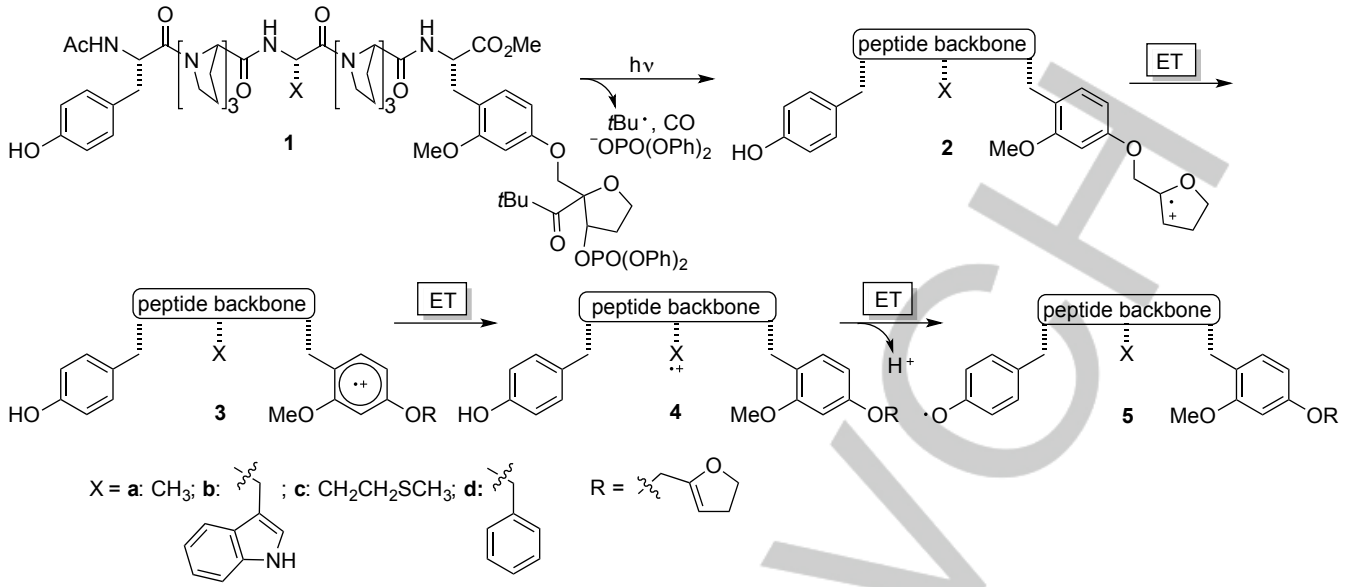
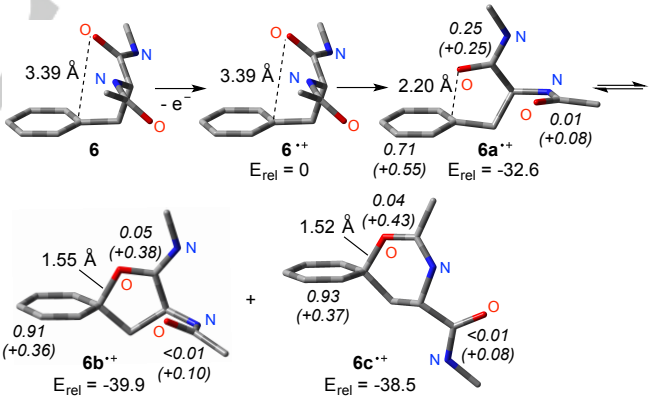


Scheme 1. Peptide assay used to study long-distance electron hole transport through peptides.

Radical cation **3**, generated by a laser flash of **1** (λ = 308 nm), acts as the electron acceptor, and Tyr at the N-terminus of the nonapeptide functions as the electron donor. At 40 ns after the laser pulse it was probed whether intermediates **4** and/or **5** could be detected by their UV/Vis spectra (Figure S1). Our experiments demonstrated that with aliphatic amino acids, which are difficult to oxidize such as Ala in **3a**, the tyrosyl radical **5a** was not formed by intramolecular ET,[10] indicating that a one-step ET is too slow to occur within 40 ns. In contrast, Trp in **3b** acts as a relay amino acid as shown by the formation of intermediates **4b** and **5b** in 30% yield (Figure S2).[10] Further studies showed that Met also increases ET rates, as we had detected the tyrosyl radical **5c** in experiments with **3c**, although a dialkyl thioether moiety has an oxidation potential of ca. 1.4 V.[14,16] Reanalysis of the laser experiments revealed now small amounts of an intermediate at 385 nm (Figure S3), which agrees with the literature value of a dialkyl thioether radical cation that is stabilized by a neighbouring pyrrolidine amide.[14] This finding indicates that neighbouring proline moieties in **1c** enable Met to function as a relay amino acid in ET processes by lowering its redox potential.[17]

Can a similar neighbouring group effect also turn Phe into a relay amino acid? Indeed, ET in **3d** generated about 15% of the tyrosyl radical **5d** within 40 ns (Figure S4). In addition, a small peak at 400 nm became visible, which is blue-shifted by 140 nm compared to the radical cation of toluene.[18] Since a hypsochromic shift of 130 nm was measured for the amide neighbouring group effect on the Met radical cation,[14] we tentatively propose that the absorption at 400 nm might be that of the Phe radical cation, which is stabilized by the neighbouring amide group. These experiments indicate that Phe could act as a stepping stone, which enables the change of the ET mechanism from superexchange to a hopping process. In future experiments the influence of amide neighbouring groups on the UV/Vis absorption of arene radical cations will be investigated in detail, using model systems similar to those of Glass and Schöneich.[14]

With the help of quantum chemical CBS-QB3//M062X/6-31G* calculations of the radical cations of Phe **6** we gained more information on a possible amide neighbouring group effect (SI). Scheme 2 shows that the neighbouring amide groups stabilize **6b**$^{\bullet+}$ and **6c**$^{\bullet+}$ by about 40 kJ mol^{-1} through formation of a covalent bond ("σ-complex").[19] The Mulliken atomic charges suggest that the stabilization is due to delocalisation of the positive charge onto the amide moiety.



Scheme 2. Stabilization of the arene radical cation of phenylalanine **6** by neighbouring group participation. Energies E_{rel} in kJ mol^{-1} (in acetonitrile) relative to **6**$^{\bullet+}$, which retains the conformation of neutral **6** (see Table S1). Italic numbers: spin densities and increases in Mulliken atomic charges from **6** (in brackets) summed up for the phenyl ring and the amide moieties.

Based on these calculations and our previous results showing that the ET intermediates **3-5** live sufficiently long (μs) to adopt the most stable conformations,[15b] our new experimental data indicate that the thermodynamic stability of arene radical cations is increased by neighbouring amide groups to such an extent that Phe could act as stepping-stone in ET through nonapeptide **3d**.

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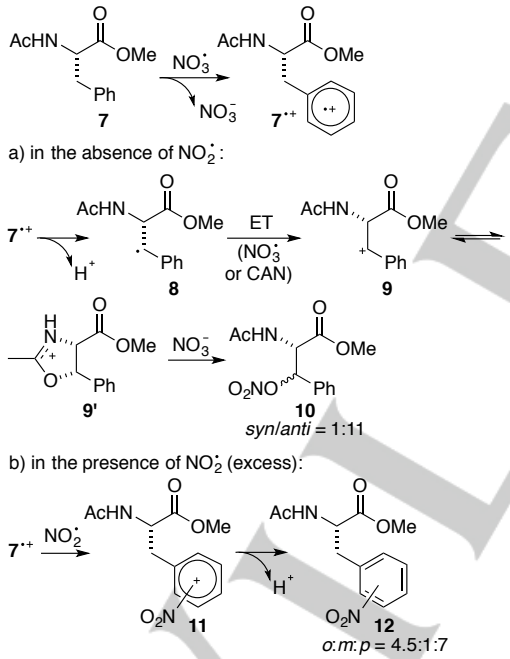
Although this neighbouring group effect may not render Phe as susceptible to oxidation as Tyr, it might reduce the oxidation potential of Phe below the critical threshold, where endergonic ET in proteins can occur with functional rates.[20,21] Thus, Phe might indeed act as a relay amino acid in pili, where it increases the ET rate by breaking down long ET steps into shorter ones.

Is it possible that such amide neighbouring group effect also speeds up the rate of intermolecular Phe oxidation? We addressed this question by studying the reaction rate of nitrate radicals with phenylalanines. $NO_3^\bullet$ is strongly oxidizing [E^o ($NO_3^\bullet/NO_3^-$) = 2.3 – 2.5 V vs. NHE],[22] and can be generated through photo-induced ET from cerium(IV) ammonium nitrate (CAN):[23]

$$(NH_4)_2Ce(NO_3)_6 + h\nu\ (355\ nm) \rightarrow NO_3^\bullet + (NH_4)_2Ce(NO_3)_5$$

We used nanosecond laser flash photolysis to determine the second-order rate coefficients k for the reaction of $NO_3^\bullet$ with Phe **7** in acetonitrile by measuring the time-dependent consumption of the $NO_3^\bullet$ signal at λ = 630 nm under pseudo-first order conditions (see SI for details).[24] In previous product studies we had shown that $NO_3^\bullet$ oxidizes the aromatic ring in Phe **7** to its arene radical cation **7**$^{\bullet+}$, which deprotonates to the benzyl radical **8** and yields, after further oxidation and recombination of the benzyl cation **9** with NO_3^-, the diastereomeric β-nitrated Phe **10** (Scheme 3a).[25,26,27]



Scheme 3. Mechanism of $NO_3^\bullet$ reactions with Phe in the absence and presence of the radical cation trap $NO_2^\bullet$.

The radical cation **7**$^{\bullet+}$ could not be detected by time-resolved spectroscopy, because the UV/Vis spectrum was dominated by the strong CAN depletion below 480 nm and the $NO_3^\bullet$ absorption at higher wavelengths (see Figure S5).[28] However, we showed previously that **7**$^{\bullet+}$ can be trapped by excess $NO_2^\bullet$ to give isomeric nitrophenylalanines **12** after deprotonation of the adduct **11** (Scheme 3b).[27,29] With aliphatic amino acids $NO_3^\bullet$ reacts through hydrogen abstraction, which is about 5-6 times slower in acetonitrile than the ET reaction with Phe **7** (not shown).[24]

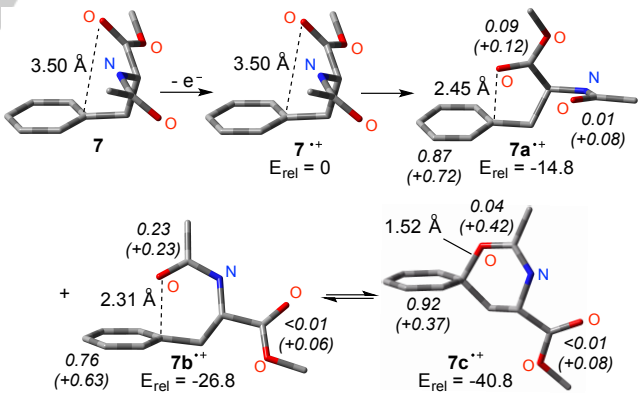
In order to elucidate how the transition state is stabilized by a neighbouring amide group, we exchanged the less nucleophilic ester group at the C-terminus in **7** by more nucleophilic amide groups in **6**, **13** and **14** (Table 1).

Table 1. Second-order rate coefficients k for the reaction of $NO_3^\bullet$ with Phe in acetonitrile at 298 ± 1 K.[a]

Substrate	k / 10^7 M^{-1} s^{-1}
Ac-Phe-OMe (**7**)	1.1
Ac-Phe-NHMe (**6**)	7.6
Ac-Phe-NH*t*Bu (**13**)	8.4[b]
Ac-Phe-NH_2 (**14**)	16

[a] Exp. error ± 10%. [b] Exp. error ± 20%.

The kinetic data revealed that the rate of $NO_3^\bullet$ consumption increased 7-15 times for amides **6**, **13** and **14**, respectively, compared to ester **7**, which shows that an amide function speeds up the oxidation of Phe. Calculations suggest that an ester stabilizes the arene radical cation **7a**$^{\bullet+}$ through a more open "π-complex" (Scheme 4), which reduces its energy by some 15 kJ mol^{-1}, compared to a radical cation **7**$^{\bullet+}$ with a peptide backbone geometry as in **7** (see Table S1). A "σ-complex" similar to **6b**$^{\bullet+}$ could not be located, clearly reflecting the lower nucleophilicity of the ester group. As a result, **7a**$^{\bullet+}$ is nearly 25 kJ mol^{-1} less stabilized than **6b**$^{\bullet+}$ relative to the radical cations in their neutral conformations (Scheme 2).



Scheme 4. Stabilization of the arene radical cation of phenylalanine **7** by neighbouring group participation. Energies E_{rel} in kJ mol^{-1} (in acetonitrile) relative to **7**$^{\bullet+}$, which retains the conformation of neutral **7** (see Table S1). Italic numbers: spin densities and increases in Mulliken atomic charges from **7** (in brackets) summed up for the phenyl ring and the amide/ester moieties.

The fast $NO_3^\bullet$ induced oxidation of Phe suggests an early transition state that is energetically close to the ground state of the neutral amino acid. In **6** and **7** the oxygen atoms of the C-

terminal carbonyl groups are only 3.4 – 3.5 Å away from the ipso carbon of the phenyl group and therefore well positioned to stabilize the developing positive charge on the aromatic ring during ET. In fact, we located the amide π-complex **6a**$^{\bullet+}$ (Scheme 2), which has a similar geometry as the ester π-complex **7a**$^{\bullet+}$ (Scheme 4), but is 18 kJ mol^{-1} more stable. In these π-complexes both charge and spin are delocalized over the ring and the amide/ester moiety, but the extent of delocalisation is considerably larger in **6a**$^{\bullet+}$ than in **7a**$^{\bullet+}$. These data suggest that the faster rate of oxidation of **6**, compared to **7**, is due to a more efficient stabilization of the developing radical cation by the C-terminal amide than by a C-terminal ester.[30] Thus, even in conformations with relatively long distances between the aromatic ipso position and the C-terminal carbonyl oxygen (2.20 – 2.45 Å) that are close to the likely transition state geometry, the amide neighbouring group has a considerable stabilizing effect. This amide neighbouring group effect is also governing oxidation reactions of peptides (Table 2).

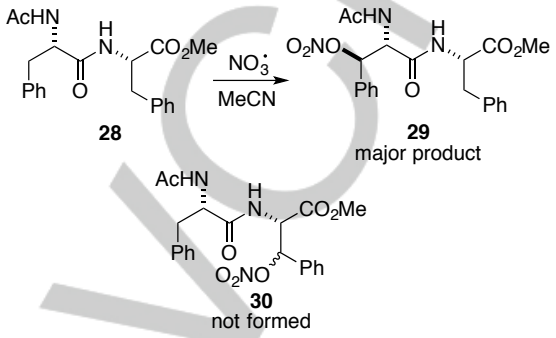
Table 2. Second-order rate coefficients *k* for the reaction of $NO_3^\bullet$ with Phe-containing di- and tripeptides in acetonitrile at 298 ± 1 K.[a]

Substrate	k / 10^7 M^{-1} s^{-1}
Ac-Leu-Phe-OMe (**15**)	1.1
Ac-Phe-Leu-OMe (**16**)	3.6
Ac-Val-Phe-OMe (**17**)	1.1
Ac-Phe-Val-OMe (**18**)	3.6
Ac-Leu-Leu-Phe-OMe (**19**)	1.9[b]
Ac-Leu-Phe-Leu-OMe (**20**)	6.3
Ac-Phe-Leu-Leu-OMe (**21**)	6.9
Ac-Val-Val-Phe-OMe (**22**)	2.3[b]
Ac-Val-Phe-Val-OMe (**23**)	6.3
Ac-Phe-Val-Val-OMe (**24**)	7.6
Ac-Phe-Leu-Phe-OMe (**25**)	7.6
Ac-Phe-Phe-Leu-OMe (**26**)	10
Ac-Phe-Phe-Val-OMe (**27**)	9.1

[a] Exp. error ± 10%. All tripeptides have low solubility in acetonitrile. [b] Exp. error ± 30%.

Di- and tripeptides **16**, **18**, **20**, **21**, **23** and **24,** where Phe has a C-terminal amide bond, are oxidized 3 times faster than the peptides **15**, **17**, **19** and **22**, where Phe is located at the ester protected C-terminus. An up to 10-fold rate enhancement was found in tripeptides **26** and **27**, where two adjacent Phe residues are flanked by amide groups and should be activated towards oxidation.

To further consolidate our findings, we performed a product study, where the Phe-Phe dipeptide **28** was reacted with $NO_3^\bullet$ (Scheme 5). The major oxidation product was dipeptide **29**, which was obtained as a single diastereomer[26] possessing a β-nitrate substituent at the Phe residue with peptide bonds in both the C- and N-direction. The isomeric dipeptide **30** with a β-nitrate ester at the less oxidizable C-terminal Phe residue was not formed (SI). This result shows that the amide neighbouring group effect not only increases the rate, but also the regioselectivity of Phe oxidation in peptides.



Scheme 5. Regioselective $NO_3^\bullet$ induced oxidation of the N-terminal Phe residue in dipeptide Ac-Phe-Phe-OMe (**28**).

To conclude, we have provided compelling evidence that Phe oxidation in peptides is facilitated through an amide neighbouring group effect, which makes Phe a relay amino acid for electron hole transfer through peptides. This amide neighbouring group effect enables a better understanding of the mechanism of EET through pili of anaerobic bacteria, such as the *Geobacter* and the *Shewanella* families. This finding is significant, since these bacteria are nowadays successfully applied for the bioremediation of radionuclide contamination in wastewater, in particular the bioreduction of U(VI) to insoluble U(IV),[4] and are also explored as electron donors in microbial fuel cells.[5] The markedly strong stabilization of the arene radical cation by primary amide groups (see **14**) suggests that amide side chains in glutamine and asparagine should provide similar accelerating effects to the reversible and irreversible oxidation of Phe. Indeed, such stabilizing interactions could be possible in the pili proteins, where a glutamine residue (Glu^{23}) is located adjacent to Phe^{24}.[12]

Acknowledgements

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Keywords: DFT calculations • electron transfer • kinetic studies • peptides • radical cations

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[30] Stabilization of the developing positive charge by the N-terminal carbonyl groups on the way to **6c**$^{\bullet+}$ and **7c**$^{\bullet+}$ is also possible after conformational changes of the peptide backbone (shown exemplarily for the π-complex **7b**$^{\bullet+}$; see also Table S1). Since the N-terminal amide is present in **6** and **7**, the energy gained through this pathway is the same in both systems.

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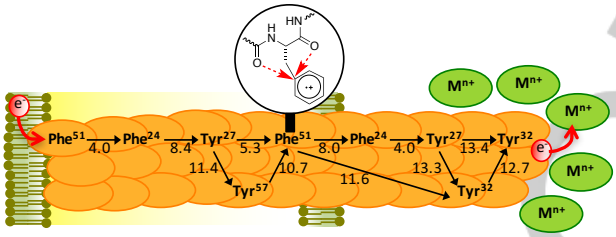
The rate of Phe oxidation in peptides is strongly modulated by the lone pairs of amide carbonyl groups, which stabilize the arene radical cation through neighbouring group effects and facilitate oxidation of the aromatic ring. This finding shows that Phe could act as a very efficient stepping stone to mediate long-distance electron hole transport in peptides.



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