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

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In nature, proteins serve as media 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 stabilise the Phe radical cation. This neighbouring-amide-group effect helps improve understanding of the mechanism of extracellular electron transfer through conductive protein filaments (pili) of anaerobic bacteria during mineral respiration.

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 by using Tyr and Phe as relay amino acids. Orange "footballs" are peptides that aggregate to the pili. Interaromatic distances [Å] for the Asp2 pilus (cell constituents are omitted for clarity).[12]

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 has become 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). Anaerobic bacteria capable of EET are typically of the *Geobacter* and *Shewanella* families.[2,3] These bacteria are used nowadays for the remediation of water, to remove toxic heavy metal and radionuclide contaminants such as Cr^{VI}, U^{VI} or Tc^{VII},[4] for example, 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 media for EET, and Lovley has observed that these protein filaments transfer electrons
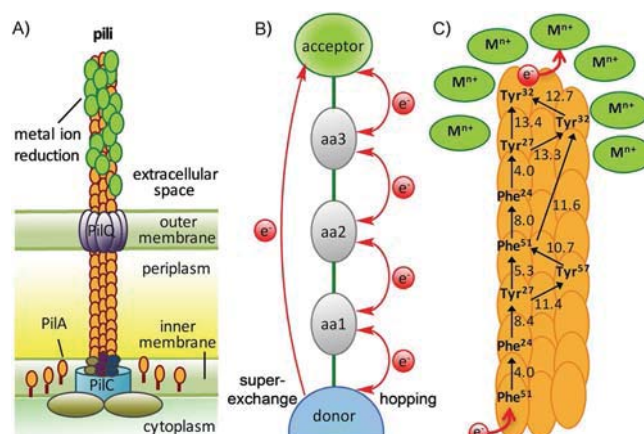
even in the absence of iron-containing cofactors (Figure 1 A).[7] Thus, ET over several hundred ångström occurs through metal-free proteins, which raises the question of the mechanism by which such EET can take place. It was recently suggested that ET through proteins and peptides over 20 Å requires a multi-step process[8] that uses the side chains of relay amino acids as stepping stones. These stepping stones are reversibly oxidised to radical or radical cation intermediates, which break up one long ET step into several short ones (Figure 1 B). 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 oxidisable. Tyr, Trp and Cys have redox potentials of around 1 V versus normal hydrogen electrode (NHE), and both kinetic and spectroscopic experiments have demonstrated the formation of short-lived intermediates during ET under oxidative conditions.[1f,8,10]

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 1 C). The redox potential of alkylated phenyl groups is about 2 V versus NHE,[13] so this suggestion is astounding and raises the question of whether Phe is more easily oxidisable in a peptide environment. Glass and Schö-
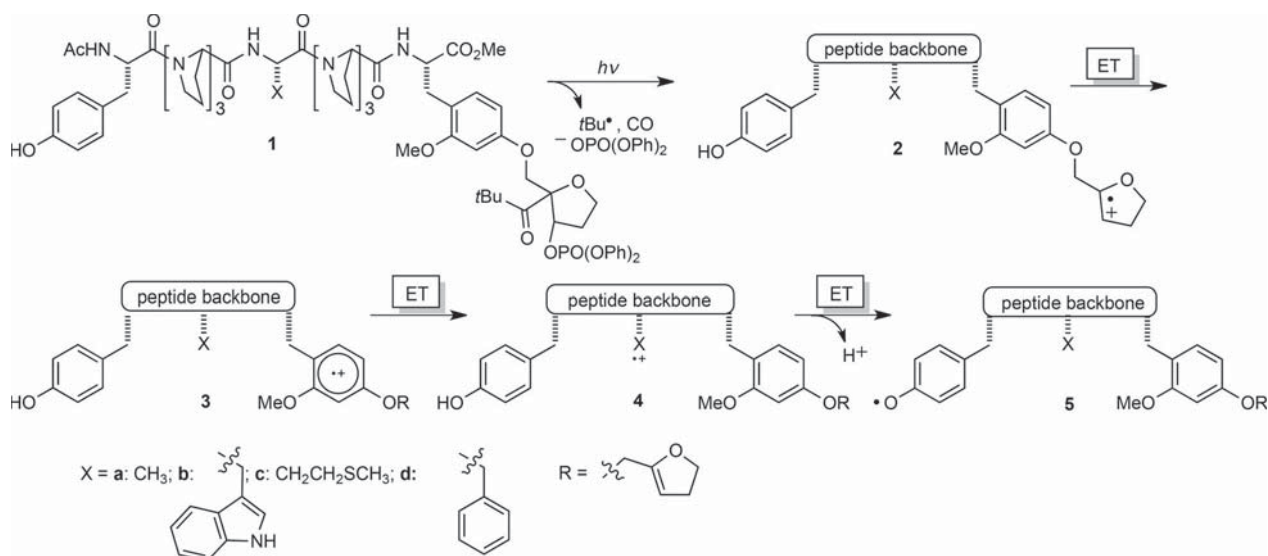
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Supporting information and the ORCID identification numbers for the authors of this article can be found under https://doi.org/10.1002/cbic.201800098.

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

neich, for instance, showed in recent pulse radiolysis experiments that the redox potentials 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]

To determine the influence of amino acids on ET rates and mechanisms, we have previously developed an assay based on nonapeptide **1** (Scheme 1).[10,15]

Radical cation **3**, generated by a laser flash of **1** ($\lambda = 308$ nm), acts as the electron acceptor, and the Tyr residue at the N terminus of the nonapeptide functions as the electron donor. At 40 ns after the laser pulse it was examined whether intermediates **4** and/or **5** could be detected by their UV/Vis spectra (Figure S1 in the Supporting Information). Our experiments demonstrated that with aliphatic amino acids, which are difficult to oxidise, such as Ala in **3a**, the tyrosyl radical **5a** was not formed by intramolecular ET,[10] thus 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, because we detected the tyrosyl radical **5c** in experiments with **3c**, although a dialkyl thioether moiety has an oxidation potential of about 1.4 V.[14,16] Reanalysis of the laser experiments now revealed small amounts of an intermediate at 385 nm (Figure S3), and this agrees with the literature value for a dialkyl thioether radical cation that is stabilised 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; this is blue-shifted by 140 nm in relation to the radical cation of toluene.[18] Because a hypsochromic shift of 130 nm was measured for the amide neighbouring-group effect on the Met radical cation,[14] we ten-

tatively propose that the absorption at 400 nm might be that of the Phe radical cation, stabilised by the neighbouring amide group. These experiments indicate that Phe could act as a stepping stone, enabling a change in 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 by using model systems similar to those of Glass and Schöneich.[14]

With the help of quantum chemical CBS-QB3//M062X/6–31G* calculations for the radical cations of Phe (**6**) we gained more information on a possible amide neighbouring-group effect (Supporting Information). Figure 2 shows that the neighbouring amide groups stabilise **6b·+** and **6c·+** by about 40 kJ mol^{-1} through the formation of a covalent bond ("σ-complex").[19] The Mulliken atomic charges suggest that the stabili-
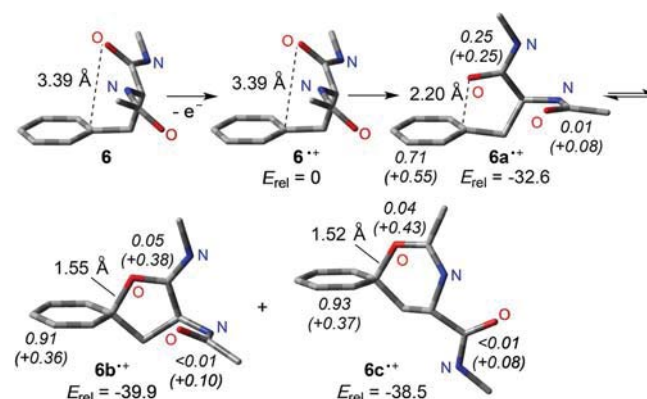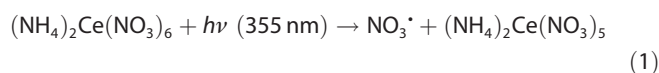

Figure 2. Stabilisation of the arene radical cation of phenylalanine (**6**) through neighbouring-group participation. Energies E_{rel} [kJ mol^{-1}] (in acetonitrile) relative to **6·+**, which retains the conformation of neutral **6** (Table S1). Italic numbers: spin densities and increases in Mulliken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

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sation is due to delocalisation of the positive charge onto the amide moiety.

In view of these calculations and of our previous results showing that the ET intermediates **3**–**5** live sufficiently long (milliseconds) 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 a stepping stone in ET through nonapeptide **3d**. Although this neighbouring-group effect might not render Phe as susceptible to oxidation as Tyr, it might reduce the oxidation potential of Phe below the critical threshold, under which endergonic ET in proteins can occur with functional rates.[20,21] Thus, Phe might indeed act as a relay amino acid in pili, in which it might increase the ET rate by breaking down long ET steps into shorter ones.

Is it possible that such an amide neighbouring-group effect might also speed up the rate of intermolecular Phe oxidation? We addressed this question by studying the reaction rates of nitrate radicals with phenylalanine derivatives. $NO_3^{\bullet}$ is strongly oxidising $[E^{\circ}(NO_3^{\bullet}/NO_3^{-}) = 2.3\text{–}2.5\ V$ vs. NHE][22] and can be generated through photoinduced ET from cerium(IV) ammonium nitrate [CAN;[23] Eq. (1)]

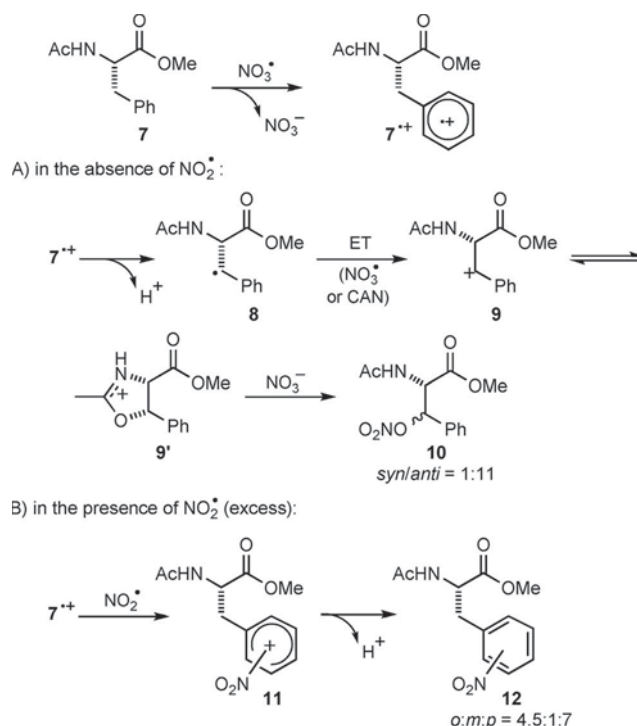$$(NH_4)_2Ce(NO_3)_6 + h\nu\ (355\ nm) \rightarrow NO_3^{\bullet} + (NH_4)_2Ce(NO_3)_5$$

(1)

We used nanosecond laser flash photolysis to determine the second-order rate coefficients (k) for the reaction between $NO_3^{\bullet}$ and Ac-Phe-OMe (**7**) in acetonitrile by measuring the time-dependent consumption of $NO_3^{\bullet}$ (signal at $\lambda = 630\ nm$) under pseudo-first order conditions (for details see the Supporting Information).[24] In previous product studies we had shown that $NO_3^{\bullet}$ oxidises the aromatic ring in Ac-Phe-OMe (**7**) to its arene radical cation **7**•+, 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 derivative **10** (Scheme 2 A).[25,26,27]

The radical cation **7**•+ could not be detected by time-resolved spectroscopy because the UV/Vis spectrum was dominated by the strong CAN depletion below 480 nm and by the $NO_3^{\bullet}$ absorption at higher wavelengths (Figure S5).[28] However, we have shown previously that **7**•+ can be trapped by excess $NO_2^{\bullet}$ to give isomeric nitrophenylalanine derivatives **12** after deprotonation of the adducts **11** (Scheme 2 B).[27,29] With aliphatic amino acids $NO_3^{\bullet}$ reacts through hydrogen abstraction, which is about five to six times slower in acetonitrile than the ET reaction with Ac-Phe-OMe (**7**; not shown).[24]

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

The kinetic data revealed that the rate of $NO_3^{\bullet}$ consumption was increased by seven to 15 times for amides **6**, **13** and **14**, in relation to that for ester **7**; this shows that an amide function speeds up the oxidation of Phe. Calculations suggest that an ester stabilises the arene radical cation **7a**•+ through a more

Scheme 2. Mechanism of $NO_3^{\bullet}$ reactions with Ac-Phe-OMe (**7**) in the absence and in the presence of the radical cation trap $NO_2^{\bullet}$.

Table 1. Second-order rate coefficients (k) for the reactions between $NO_3^{\bullet}$ and Phe derivatives in acetonitrile at (298 ± 1) K.[a]			
Substrate	$k\ [10^7\ M^{-1}\ s^{-1}]$	Substrate	$k\ [10^7\ M^{-1}\ s^{-1}]$
Ac-Phe-OMe (**7**)	1.1	Ac-Phe-NH*t*Bu (**13**)	8.4[b]
Ac-Phe-NHMe (**6**)	7.6	Ac-Phe-NH$_2$ (**14**)	16
[a] Exp. error ±10%. [b] Exp. error ±20%.			

open "π-complex" (Figure 3), which reduces its energy by some 15 kJ mol^{-1} relative to that of a radical cation **7**•+ with
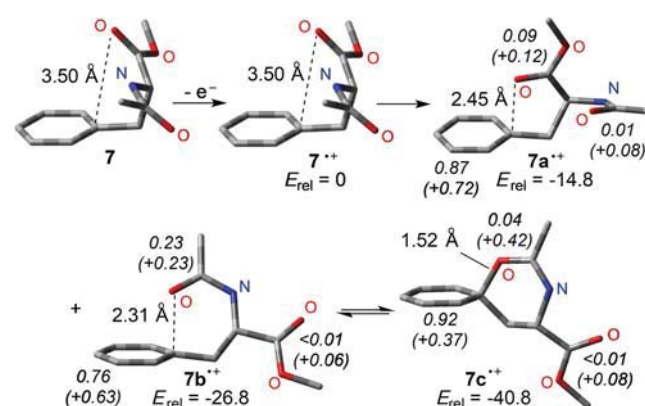

Figure 3. Stabilisation of the arene radical cation of Ac-Phe-OMe (**7**) through neighbouring-group participation. Energies E_{rel} [kJ mol^{-1}] (in acetonitrile) relative to **7**•+, which retains the conformation of neutral **7** (Table S1). Italic numbers: spin densities and increases in Mulliken atomic charges from **7** (in brackets) summed up for the phenyl ring and the amide/ester moieties.

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the same peptide backbone geometry as in **7** (Table S1). No "σ-complex" similar to **6b**[•+] could be located; this clearly reflects the lower nucleophilicity of the ester group. As a result, **7a**[•+] is nearly 25 kJ mol⁻¹ less stabilised than **6b**[•+] relative to the radical cations in their neutral conformations (Figure 2).

The fast NO₃[•]-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 atom of the phenyl group and therefore well positioned to stabilise the developing positive charge on the aromatic ring during ET. In fact, we located the amide π-complex **6a**[•+] (Figure 2), which has a geometry similar to that of the ester π-complex **7a**[•+] (Figure 3), but is 18 kJ mol⁻¹ more stable. In these π-complexes both charge and spin are delocalised over the ring and the amide/ester moiety, but the extent of delocalisation is considerably larger in **6a**[•+] than in **7a**[•+]. These data suggest that the faster rate of oxidation of **6**, relative to **7**, is due to more efficient stabilisation 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 stabilising effect. This amide neighbouring-group effect also governs oxidation reactions of peptides (Table 2).
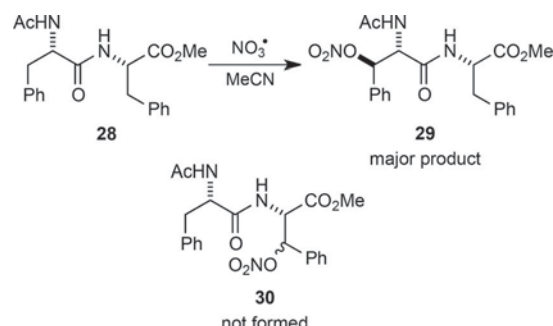
Table 2. Second-order rate coefficients (*k*) for the reactions between NO₃[•] and Phe-containing di- and tripeptides in acetonitrile at (298 ± 1) K.[a]

Substrate	k [10⁷ M⁻¹ s⁻¹]	Substrate	k [10⁷ M⁻¹ s⁻¹]
Ac-Leu-Phe-OMe (**15**)	1.1	Ac-Val-Val-Phe-OMe (**22**)	2.3[b]
Ac-Phe-Leu-OMe (**16**)	3.6	Ac-Val-Phe-Val-OMe (**23**)	6.3
Ac-Val-Phe-OMe (**17**)	1.1	Ac-Phe-Val-Val-OMe (**24**)	7.6
Ac-Phe-Val-OMe (**18**)	3.6	Ac-Phe-Leu-Phe-OMe (**25**)	7.6
Ac-Leu-Leu-Phe-OMe (**19**)	1.9[b]	Ac-Phe-Phe-Leu-OMe (**26**)	10
Ac-Leu-Phe-Leu-OMe (**20**)	6.3	Ac-Phe-Phe-Val-OMe (**27**)	9.1
Ac-Phe-Leu-Leu-OMe (**21**)	6.9		

[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**, in which Phe has a C-terminal amide bond, are oxidised three times more rapidly than the peptides **15**, **17**, **19** and **22**, in which Phe is located at the ester-protected C terminus. Rate enhancement of up to tenfold was found in tripeptides **26** and **27**, in which two adjacent Phe residues are flanked by amide groups and should be activated towards oxidation.

To consolidate our findings further, we performed a product study in which the Phe-Phe dipeptide **28** was treated with

Scheme 3. Regioselective NO₃[•]-induced oxidation of the N-terminal Phe residue in dipeptide Ac-Phe-Phe-OMe (**28**).

NO₃[•] (Scheme 3). 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 the N-direction. The isomeric dipeptide **30** with a β-nitrate ester at the less oxidisable C-terminal Phe residue was not formed (Supporting Information). This result shows that the amide neighbouring-group effect increases not only the rate but also the regioselectivity of Phe oxidation in peptides.

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 because 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 stabilisation of the arene radical cation by primary amide groups (see **14**) suggests that amide side chains in glutamine and asparagine should provide accelerating effects similar to those seen in the reversible and irreversible oxidation of Phe. Indeed, such stabilising interactions could be possible in the pili proteins, in which a glutamine residue (Glu²³) is located adjacent to Phe²⁴.[12]

Conflict of Interest

The authors declare no conflict of interest.

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[25] The sequential ET/benzylic deprotonation mechanism, as opposed to direct benzylic hydrogen abstraction, was shown by the absence of an H/D isotope effect [$NO_3^{\cdot}$+Ac-(β,β-D_2)Phe-OMe: $k = 1.0 \times 10^7\ M^{-1}\,s^{-1}$, 298 K, in acetonitrile].

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