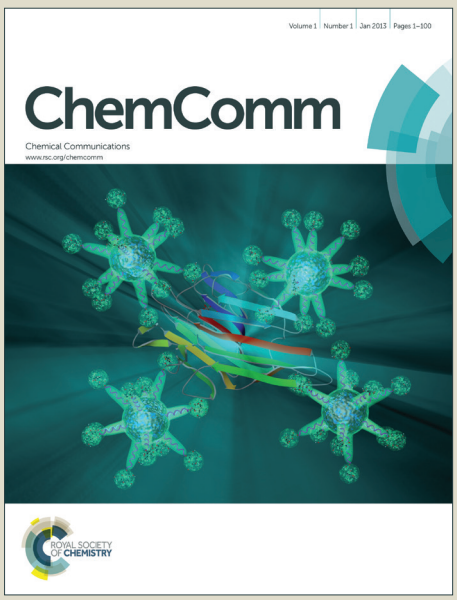
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

RSCPublishing

COMMUNICATION

Carbon-Centered Scavenger Radicals Add Reversibly to Histidine – Implications

Cite this: DOI: 10.1039/x0xx00000x

Thomas Nauser[a]* and Anna Carreras[a]

Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Carbon-centered radicals of alcohols commonly used as hydroxyl radical scavengers (MeOH, EtOH, *i*-PrOH and *t*-BuOH) add reversibly to histidine with equilibrium constants up to 3×10^3 M^{-1} and rate constants on the order of 10^9 $M^{-1}s^{-1}$. Similar equilibria may compromise determinations of one-electron (radical) electrode potentials.

Radicalic addition to aromatic substrates is well known in preparative chemistry[1] but in biochemistry and pulse radiolysis of aqueous solutions, it has drawn little attention. For aromatic amino acids, investigations usually focus on electron transfer. Tyrosyl and tryptophanyl radicals are comparably stable, have distinct UV-Vis spectra and are therefore easy to detect.[2,3] They have lower electrode potentials (≈1 V)[4] than other amino acid radicals and therefore are considered the thermodynamic sinks of intramolecular electron transfer in peptides. Importantly, *in vivo*, they may be repaired by endogenous antioxidants before irreversible protein damage occurs.[5,6] Addition of the primary radiolysis radicals e^-_{aq}, $H^\bullet$ and $HO^\bullet$ to aromatic moieties is known, and we have reported that thiyl radicals add to phenylalanine to form cyclohexadienyl radicals.[7] Now we find that also carbon-centered radicals add to aromatic amino acids. To demonstrate this, we chose His as the aromatic reaction partner: it is not easily oxidized by one electron ($E^{\circ\prime}$(pH 7) = +1.2 V)[8] and has negligible absorption above 230 nm. Thus, electron-transfer is unlikely to occur and products can be easily distinguished from reactants spectroscopically. As reaction partners, we use carbon-centered radicals of alcohols (MeOH, EtOH, *i*-PrOH and *t*-BuOH). All four absorb weakly at $\lambda > 320$ nm and are perceived as unreactive, with exception of recombination reactions.

Qualitatively, an adduct of carbon-centered radicals to His can be shown by the absorption spectra of pulse-irradiated solutions that contain His and a concentration of *t*-BuOH high enough to warrant quantitative scavenging of $HO^\bullet$ (Fig. 1). Any absorption above 280 nm is due to a product, or products, with His: *t*-$^\bullet$BuOH radicals have a very low absorptivity here[9] and the primary radicals are very short lived. Known chemistry is governed by reactions (1) – (3) and, if solutions contain N_2O, reaction (4).

$$e^-_{aq} + His + H_2O \longrightarrow [His^{\cdot\cdot}H]^\bullet + HO^- \quad (1)$$

$$H^\bullet + His \longrightarrow [His^{\cdot\cdot}H]^\bullet \quad (2)$$

$$HO^\bullet + t\text{-BuOH} \longrightarrow {}^\bullet CH_2C(CH_3)_2OH\ (t\text{-}{}^\bullet BuOH) + H_2O \quad (3)$$

$$e^-_{aq} + N_2O + H_2O \longrightarrow N_2 + HO^\bullet + HO^- \quad (4)$$

We irradiate our solutions for 50 ns with 2 MeV electrons†, and use the known radiation chemical yields, or G-values#, to interpret our results. In water, G = 1 means formation of 0.1036 µM product per Gy.

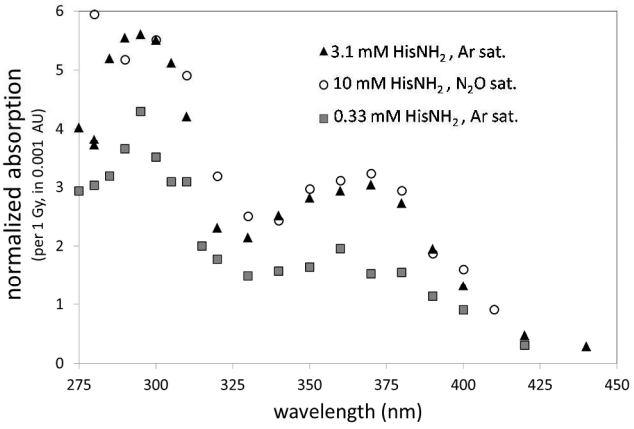


Fig. 1 Spectra of aqueous solutions containing $HisNH_2$ and 1 M t-BuOH 2 µs after irradiation (50 Gy, optical pathlength 6 cm). Black triangles: 3.1 mM $HisNH_2$, Ar saturated; open circles: 10 mM HisNH2, N_2O saturated; grey squares: 0.33 mM $HisNH_2$, Ar saturated

The spectrum of an irradiated, Ar-saturated solution of 3.1 mM $HisNH_2$ and 1 M *t*-BuOH has maxima at 290 nm and 360 nm (Fig. 1, black triangles) which usually are assigned solely to the adduct of the hydrogen atom to histidine (reactions (1) and (2)).[10,11] We expect $G([His^{\cdot\cdot}H]^\bullet) = G(e^-_{aq}) + G(H^\bullet) = 2.75 + 0.55 = 3.3$. Upon irradiation of a

N_2O-saturated solution of 10 mM $HisNH_2$ and 1 M *t*-BuOH, the solvated electrons will also react with N_2O, reaction 4. Here, we expect G([His···H]•) = 0.8,‡ given the competition between N_2O and $HisNH_2$ for the solvated electron (reactions (1) and (4)), and the quantitative reaction of H• with $HisNH_2$ (reaction (2)). Thus, the yield of [His···H]• is 4 times lower in the N_2O-saturated solution than the Ar-saturated solution. However, the absorbance changes are small (Fig. 1, open circles and black triangles). Therefore, the spectra cannot be attributed to [His···H]• only and we have to accept that *t*-•BuOH radicals *also* add to $HisNH_2$ (reaction (5)).

$$t\text{-}^{\bullet}BuOH + HisNH_2 \rightleftharpoons [HisNH_2^{\cdots}t\text{-}BuOH]^{\bullet} \text{ (adduct 1)} \quad (5)$$

If the reverse reaction (-5) was negligible, pulse-irradiation of an Ar saturated solution of 0.33 mM HisNH2 in 1 M t-BuOH (Fig.1, grey squares) should show essentially the same spectrum as with 3.1 mM HisNH2 (Fig.1, black triangles). This is not the case. Likewise, the initial product absorption, caused by adduct 1 (reaction (5)), of N_2O–saturated solutions is dependent on the $HisNH_2$ concentration (Fig 2). The dependence is clearly non-linear. For those reasons, we believe adduct 1 to be in equilibrium with its reactants, and assume a reaction sequence dominated by reactions (3), (5), (-5) and (6), where the rate of equilibration (reactions (5) and (-5)) is much faster than the rate of radical recombination (reaction (6)).

$$2\ t\text{-}^{\bullet}BuOH \rightarrow \text{products (non – absorbing)} \quad (6)$$

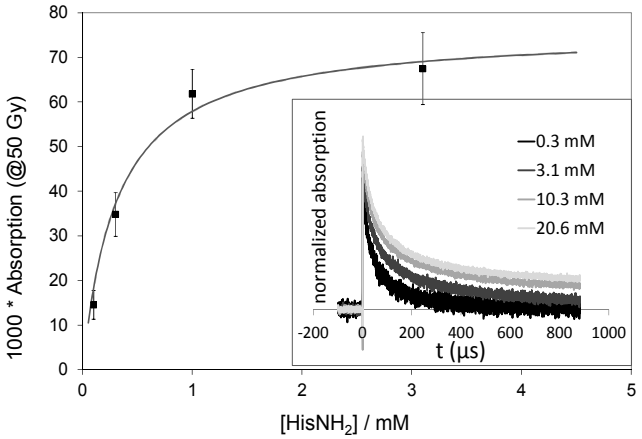


Fig. 2 Absorption at 360 nm 5 µs after irradiation (50Gy) of a N_2O saturated aqueous solutions of 1 M *t*-BuOH containing variable amounts of $HisNH_2$. The curve represents a fit for K_5=3×10^3 M^{-1} and ε_{360nm}([$HisNH_2^{\cdots}t$-BuOH]•) = 4 × 10^2 M^{-1}cm^{-1}. Inset: Kinetics traces normalized to initial absorption. The decay slows down with increasing concentrations of $HisNH_2$

It follows mathematically, that [$HisNH_2^{\cdots}t$-•BuOH] (adduct 1) is proportional to [*t*-•BuOH] at all [$HisNH_2$] (equilibrium (5)), as in all experiments [$HisNH_2$] >> [*t*-•BuOH]. What we observe seems analogous to a complex formation between an electrophile, in our case the radical, and a ligand, His: formation of adduct 1 reaches a maximum asymptotically (Fig. 2) and the decay rate of adduct 1 decreases with increasing concentration of $HisNH_2$ (Fig. 2, inset). We expect this because the equilibrium concentration of *t*-•BuOH is decreased and radical recombination (reaction (6)) proceeds more slowly. We obtain an equilibrium constant K_5 of 3×10^3 M^{-1}. In the presence of 0.33 mM HisNH2, formation of adduct 1 has a half-live distinctly smaller than 1 µs. With $k_{obs} = k_5[HisNH_2] + k_{-5} = \ln(2)/t_{½}$ we calculate that $k_5 \geq 10^9$ M^{-1}s^{-1}. Therefore reaction (5) is one order of magnitude faster than reaction (2).[12] At concentrations of histidine higher than 3 mM, we observe phenomena which may be due to aggregation, *i.e.* micelle formation or π-stacking: there is an additional increase in absorptivity with the histidine concentration (Fig. S1, supplementary information), and the mechanism of the decay seems to change (Fig. S2, supplementary information).

Similar results were obtained with solutions of AcHis at pH 7.† Smaller equilibrium constants are found for the reaction of the α-hydroxyalkyl radicals (•CH_2OH, CH_3•$CHOH$ and CH_3•$C(OH)CH_3$) with histidine (Table 1)). In contrast to *t*-•BuOH radicals, they all contribute to the absorption at λ<320 nm, therefore quantification is carried out only for λ = 360 nm. Furthermore, α-hydroxyalkyl radicals are potent reductants –(0.9V – 1.5V).[4] Unlike *t*-BuOH, primary and secondary alcohols do react quickly with H-atoms,[13] and because they are present in three orders of magnitude excess over His, H-atoms are scavenged quantitatively by those alcohols: here the contribution of [His···H]• to the overall absorption is negligible. In all cases, a broad absorption band with a maximum around 360 nm and a sharper, stronger band at approximately 300 nm are found (for •CH_2OH, see Fig. S3, supplementary information). The molar absorptivities are comparable. This is expected if indeed all these chromophores stem from addition of a radical to $HisNH_2$. Two UV-absorption bands are also reported for the product of the reaction of HO• radicals with His.[10]

Preliminary experiments with the other aromatic amino acids indicate the existence of similar addition equilibria. We are therefore confident that radical addition equilibria to aromatic residues is a general phenomenon. In line with our observations, •CH_2OH adds to pyrimidine nucleobases, but with a rate constant five orders of magnitude lower than those obtained here.[14]

Table 1: Equilibrium constants for the addition of carbon-centered radicals to histidine (in M^{-1})

	•$CH_2C(CH_3)_2OH$	•CH_2OH	CH_3•$CHOH$	CH_3•$C(OH)CH_3$
AcHis	2 × 10^3	2 × 10^2	~10^2	< 10^2
$HisNH_2$	3 × 10^3	3 × 10^2	-	-

Pulse radiolysis experiments have been one of the most important sources for electrode potentials of radicals.[4] Usually, they are based on simple equilibria

$$A^{\bullet} + BH \rightleftharpoons AH + B^{\bullet} \quad (7)$$

If the electrode potential $E°(A^{\bullet},H^{+}/AH)$ is known and K_7 can be determined, then

$$E°(B^{\bullet},H^{+}/BH) = E°(A^{\bullet},H^{+}/AH) - (RT/F) \times \ln K_7 \quad (8)$$

With the applied dose also the total radical concentration is known. If either A• or B• can be quantified, *K7* is easily calculated from the starting conditions. Many of the reference compounds used are aromatic molecules because their radicals exhibit high molar

absorptivities and are easy to quantify. As shown here, the reaction may also proceed as

$$A^{\bullet} + BH \rightleftharpoons [A^{\cdots}BH]^{\bullet} \quad (9)$$

$$[A^{\cdots}BH]^{\bullet} \rightleftharpoons AH + B^{\bullet} \quad (10)$$

For example, upon one-electron oxidation, the widely used redox indicator $ABTS^{2-}$ is known *not* to yield the expected $ABTS^{\bullet-}$ radicals quantitatively. The yields even depend on the oxidant.[15] This can be rationalized by radical addition (reaction 9). Equation (8) is valid only if $[A^{\cdots}BH]^{\bullet}$ does not interfere with the measurement, i.e. equation (9) can be neglected. If equilibria involving radical addition are widespread, then they may have a serious impact on the validity of published values for one-electron electrode potentials. The determinations of the electrode potential of Tyr and Trp have a considerable scatter.[16,17] We suspect the involvement of adduct reactions.

In 2013, more than 700 papers were published on oxidative radical damage and radical repair in biochemistry, biology, food technology and in medical care. Known oxidative post-translational modifications include non-peptidic bonds between His and Cys (tyrosinase, hemocyanin, catechol oxidase) and Tyr and Cys (galactose oxidase, cysteine dioxygenase).[18] An explanation that is often found is that these bonds are formed *via* the concomitant production of two adjacent radicals. This is most improbable under biological settings. Radical addition, followed by oxidation, provides an alternative explanation. The primary oxidant, for example FeO^{2+}, is generally a high-valent metal species which can easily produce an amino acid radical, such as a thiyl radical. Then, in analogy to reaction (5), an adduct is formed with an aromatic residue. Subsequent oxidation by the metal center or oxygen creates a covalent non-peptidic bond between Cys and Tyr or His. Similarly, a product with a covalent bond between glutathione and tyrosine was found.[19] Preliminary data indicate that thiyl radicals add to His and Tyr like they add to Phe. Radical addition could also be the underlying mechanism in the photoaggregation of the eye-lens protein γD-crystallin, which is critically dependent on neighboring Cys(18) and Tyr(16) that cooperatively cause the formation of new bonds.[20] An analogous case can be made for cytochrome *c* oxidase, where a covalent bond between Tyr and His is found near haem a_3 and Cu_B.[18]

If the addition of radicals to aromatic structures, in our case histidine, is indeed fast and reversible, then the one-electron oxidation of the radical adduct is the limiting factor for the yield and the rate-determining step in the formation of stable products both in preparative chemistry and in biochemistry.

Notes and references

[a] Laboratorium für Anorganische Chemie, Departement für Chemie und Angewandte Biowissenschaften, ETH Zürich, Vladimir-Prelog Weg 2, CH-8093 Zürich, Switzerland. e-mail: nauser@ethz.ch

† Experiments were carried out at the pulse radiolysis facility of ETH.[9] The optical pathlength of the quartz cell is 6 cm. Dosimetry was carried out with thiocyanate and ferrocyanide.[21] The solutions contained $HisNH_2 \cdot HCl$ or AcHis from Bachem (Switzerland). Solutions containing $HisNH_2$ were prepared without buffer, solutions of AcHis were prepared from stock solutions of 10 mM AcHis with 15 mM K_2HPO_4 with pH = 7. MilliQ water (18.2 MΩ , Millipore, Zug (Switzerland)) was used. The alcohols were of the highest available purity, tert-butanol was recrystallised multiple times.

\# in molecules produced per 100eV absorbed dose.

‡ Given the rate constants $k_1 = 1.7 \times 10^9\ M^{-1}s^{-1}$, (pH 7)[11] $k_4 = 9.1 \times 10^9\ M^{-1}s^{-1}$,[13] and a saturation concentration of N_2O of 24 mM we calculate that 7% of the electrons react with $HisNH_2$. That results in $G(\text{reaction 1}) \approx 0.24$

Support by the ETH is gratefully acknowledged. We thank W.H. Koppenol for valuable discussions.

1. Minisci, F., Fontana, F., and Vismara, E., *J.Heterocylic Chem.*, 1-1-1990, **27**, 79-96.
2. Land, E. J. and Ebert, M., *Transactions of the Faraday Society*, 1967, **63**, 1181-1190.
3. Santus, R. and Grossweiner, L. I., *Photochem.Photobiol.*, 1972, **15**, 101-105.
4. Wardman, P., *J.Phys.Chem.Ref.Data*, 1989, **18**, 1634-1755.
5. Domazou, A., Gebicki, J. M., Nauser, T., and Koppenol, W. H., *Isr.J.Chem*, 2014, **54**, 254-264.
6. Gebicki, J. M., Nauser, T., Domazou, A., Steinmann, D., Bounds, P. L., and Koppenol, W. H., *Amino Acids*, 2010, **39**, 1131-1137.
7. Nauser, T., Casi, G., Koppenol, W. H., and Schöneich, C., *Chem.Comm.*, 2005, 3400-3402.
8. Navaratnam, S. and Parsons, B. J., *J.Chem.Soc.Faraday Trans.*, 1998, **94**, 2577-2581.
9. Nauser, T., Casi, G., Koppenol, W. H., and Schöneich, C., *J.Phys.Chem.B*, 2008, **112**, 15034-15044.
10. Rao, P. S., Simic, M., and Hayon, E., *J.Phys.Chem.*, 1975, **79**, 1260-1263.
11. Faraggi, M. and Bettelheitm, A., *Radiat.Res.*, 1977, **71**, 311-324.
12. Neta, P. and Schuler, R. H., *J.Phys.Chem.*, 1972, **76**, 2673-2679.
13. Buxton, G. V., Greenstock, C. L., Helman, W. P., and Ross, A. B., *J.Phys.Chem.Ref.Data*, 1988, **17**, 531-886.
14. von Sonntag, C., *The Chemical Basis of Radiation Biology*; Taylor & Francis: London, 1987
15. Wolfenden, B. S. and Willson, R. L., *J.Chem.Soc.Perkin Trans.2*, 1982, 805-812.
16. Harriman, A., *J.Phys.Chem.*, 1987, **91**, 6102-6104.
17. DeFelippis, M. R., Murthy, C. P., Faraggi, M., and Klapper, M. H., *Biochemistry*, 1989, **28**, 4847-4853.
18. Okeley, N. M. and van der Donk, W. A, *Chemistry and Biology*, 2000, **7**, 159-171.
19. Folkes, L. K., Trujillo, M., Bartesaghi, S., Radi, R., and Wardman, P., *Arch.Biochem.Biophys.*, 15-2-2011, **506**, 242-249.
20. Schafheimer, N, Wang, Z., Schey, K., and King, J., *Biochemistry*, 2014, **53**, 979-990.
21. Schuler, R. H., Patterson, L. K., and Janata, E., *J.Phys.Chem.*, 1980, **84**, 2088-2089.