Characterization of the Site-Specific Acid-Base Equilibria of 3-Nitrotyrosine

Tamás Pálla, a Erzsébet Fögarasi, b Béla Noszál, *a and Gergő Tóth *a

a Semmelweis University, Department of Pharmaceutical Chemistry, Hőgyes E. u. 9, 1092 Budapest, Hungary, e-mail: noszal.bela@pharma.semmedweis-univ.hu; toth.gergo@pharma.semmedweis-univ.hu

b University of Medicine, Pharmacy, Sciences and Technology of Târgu Mureș, Faculty of Pharmacy, Department of Toxicology and Biopharmacy, Str. Marinescu Gheorghe 38, 540142 Târgu Mureș, Romania

© 2019 The Authors. Published by Wiley-VHCA AG. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

The complete macro- and microequilibrium analyses of 3-nitrotyrosine, a biomarker of oxidative stress damage, are presented for the first time. The protonation macroconstants were determined by 1H-NMR-pH titration, while microconstants were elucidated by a combination of deductive and NMR methods, in which properties of the methyl ester derivative as an auxiliary compound were also studied. Combination of the NMR-pH characterization of the title and auxiliary compounds and the pair-interactivity parameters of 3-iodotyrosine provided the sufficient system to evaluate all the microconstants. NMR-pH profiles, macroscopic and microscopic protonation schemes, and species-specific distribution diagrams are included. The phenolate basicity of 3-nitrotyrosine is 500 times below that of tyrosine, and it is even lower than that of 3-iodotyrosine. This phenomenon can be explained by the stronger electron withdrawing and the negative mesomeric effect of the nitro group. Based on our results, 89% of the phenolic OH groups are deprotonated in 3-NT molecules at the pH of the blood plasma.

Keywords: nitrotyrosine, microspeciation, oxidative stress, NMR, pK_a.

Introduction

3-Nitrotyrosine (3-NT, Figure 1) is the earliest discovered biomarker of nitrosative stress. It is formed through the nitration of tyrosine (Tyr) by peroxynitrinate. Peroxynitrinate is not a free radical itself but it can become a source of free radicals by an electron accepting process. Being an isomeric form of nitrate, the lifetime of peroxynitrinate is a fraction of a second at physiological pH, thus Tyr nitration represents its most important footprint in biological systems. The formation of peroxynitrinate in organisms takes place by diffusion-controlled reaction of NO* (nitric oxide) and O_2*^- (anion superoxide), in various, enzyme-associated process,[1] starting mainly from l-arginine. Some in vivo experiments demonstrated its release from nitrones by the action of hydroxyl radical.[2] Peroxynitrinate, the deprotonated form of peroxynitrous acid can decompose to *OH and *NO_2, the latter being the most important nitration agent of Tyr.[3] Tyr nitration takes place in two steps: i) production of tyrosyl radical (Tyr*) by one-electron oxidation of the phenolic ring and ii) Tyr* and *NO_2 radical-radical coupling reaction with a rate constant of 3 \times 10^9 M^{-1}s^{-1}. Oxidants that can achieve the one-electron oxidation of Tyr are: *OH, *NO_2, CO_3^{2-}, LOO*, LO*, oxo-metal compounds (O=Mn^{n+}), myeloperoxidase (MPO). Usually, *NO_2 is responsible for solvent-exposed Tyr nitration, while transition metals can lead to nitration of buried Tyr residues.[1]

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.201900358

Figure 1. The constitutional formula of 3-nitrotyrosine.
The target of electrophilic reagents and/or oxidants in the Tyr structure is the position 3 of the aromatic ring. The nitration of Tyr in proteins in most cases leads to inactivation of the enzymes. Several properties of 3-NT and the proteins, containing it have been described. For example, at low pH, 3-NT is more hydrophobic than Tyr, while at high pH, it is more polar than Tyr.\cite{4,5} Tyr nitration obviously alters the protein structures and concomitantly the physiological effects. These modifications are strongly related to diseases, mainly with degenerative and inflammatory symptoms.

Although the role of 3-NT has been studied in oxidative stress, no previous report was devoted to its microspeciation. Therefore, our aim was to characterize the acid-base properties of 3-NT at the site-specific level. Macroscopic protonation constants ($K$) are known, based on a previous work which used capillary zone electrophoresis.\cite{6} Nevertheless, we also determined the $K$ values by $^1$H-NMR-pH titration method. Macroscopic equilibrium constants of multiprotic molecules give information only on the acid-base properties of the compound as a whole. Site-specific, submolecular basicities can be obtained when microconstants are determined. The site-specific acid-base characterization of 3-NT is therefore of fundamental importance to allow the interpretation of its biological functions at site-specific level.

Results and Discussion

3-NT is a triprotic molecule with phenolate, amino and carboxylate protonation sites. As a triprotic molecule, the total number of microspecies and microconstants are 8 and 12, respectively. The macroscopic- and microscopic protonation schemes are shown in Figure 2.

The relationships between the micro- and macroconstants which has been used for the calculation of microspeciation are as follows:

$$K_1 = k^O + k^N + k^C$$

$$\beta_2 = K_1K_2 = k^O k^N + k^O k^C + k^N k^C = k^N k^C + k^O k^C$$

$$\beta_3 = K_1K_2K_3 = k^O k^N k^C = k^N k^C$$

Equation 3 can be written in six different and equivalent ways based on the Hessian-relationship of protonation constants which means that the sum of

Figure 2. The micro- and macrospeciation schemes of 3-nitrotyrosine, where microconstants with superscript O, N and C belong to the phenolate, amino and carboxylate site, respectively, and $K_1$, $K_2$ and $K_3$ are stepwise macroconstants. The superscript on the microconstant indicates the protonating site, while the subscript (if any) stands for the site(s) already protonated.
protonation constants in logarithmic units is constant between the same start and end-points regardless of the path of protonation.

**Determination of Protonation Macroconstants**

Evaluation of the protonation constants from $^1$H-NMR-pH titration curves was based on the principle that non-exchanging NMR nuclei near the basic site sense different electronic environments upon protonation, and change their chemical shifts accordingly. All carbon-bound protons could be observed.

The protonation macroconstants of 3-NT and its methyl ester derivate were determined by investigating the chemical shift changes of the CH proton and the aromatic proton next to the nitro group in $^1$H-NMR-pH titrations (Figure 3). Since the effect of protonation on the chemical shifts diminishes along with increasing distance from the site of protonation, the aromatic proton signals were much more sensitive for the protonation of the phenolate group, while the chemical shifts of the aliphatic protons changed more upon the protonation of the amino and the carboxylate groups. Some representative NMR spectra at different pH values were depicted in the Supporting Information (Figure S1).

The protonation constants were determined by nonlinear curve fitting of Equation 19 to the collected data. The values are shown in Table 1.

The macroconstant values determined by capillary electrophoresis earlier and our values are in good agreement.

**Complete Microspeciation of 3-Nitrotyrosine**

For the determination of all microconstants, the macroconstants of 3-NT and its carboxymethyl deriva-

| Table 1. Logarithmic values of macroscopic protonation constants of 3-nitrotyrosine and methyl 3-nitrotyrosinate (mean ± SD, n = 3) |
|---------------------------------------------------------------|
| 3-Nitrotyrosine* (CZE) | 3-Nitrotyrosine | Methyl 3-nitrotyrosinate |
| logK₁ | 9.54 ± 0.01 | 9.452 ± 0.002 | 7.570 ± 0.006 |
| logK₂ | 6.82 ± 0.01 | 6.895 ± 0.003 | 6.371 ± 0.01 |
| logK₃ | 2.06 ± 0.01 | 2.154 ± 0.002 | – |

The data in column marked with * are from the capillary zone electrophoresis-pH studies of Ren et al. [6]
tive, and the pair-interactivity parameters of 3-iodotyrosine from our previous work were used.\textsuperscript{[7]}

Protonation of a basic site reduces the basicity of the other basic sites in the molecule. This basicity-reducing effect is strong if the sites are in nearby positions, but it gradually fades away along with the increasing distance. The basicity-modifying effect between two moieties can be quantified by the pair-interactivity parameter. For a triprotic molecule, three different pair-interactivity parameters are defined. For example, the interactivity parameter ($E$) in log units between the amino and the carboxylate sites is:

$$\log E^N_N = \log k^N - \log k^N_C = \log k^C - \log k^N_N = \ldots \quad (4)$$

The pair-interactivity parameter is the most invariant and best transferable parameter between molecules having analogous moieties.\textsuperscript{[8]} Due to the similarity between the nitro- and iodo-group, the pair-interactivity parameter of 3-iodotyrosine can be used in the microspeciation of 3-NT as well. The three pair-interactivity parameters were calculated in a previous work,\textsuperscript{[7]} while the standard deviations were based on the Gaussian propagation of uncertainty (Table 2).

In classical Tyr microspeciation works,\textsuperscript{[9]} UV-pH titrations were successfully used, owing to the overlapping protonations of the phenolate and amino sites, and the selective pH-dependence of the phenolate UV-absorption. For 3-NT, however, this method should not work, due to the negligible overlap between the phenolate and amino protonation regions.

Using the Hessian relationship with the macro-constants of 3-NT and methyl 3-nitrotyrosinate, $\log k^C$ was calculated as follows:

$$\log k^C = (\log k_1^O + \log k_2^O + \log k_3^O) - (\log k_1^C + \log k_2^C) \quad (5)$$

where the protonation macroconstants of 3-NT are in bold, the protonation macroconstants of methyl 3-nitrotyrosinate are in normal fonts. After the value of $\log k^C$ is determined, $\log k^C_0$, $\log k^C_N$, and $\log k^C_{O,N}$ can be calculated by using the pair-interactivity parameters and the appropriate form of Equation 4:

$$\log k^C_0 = \log k^C - \log E^{O-N} \quad (6)$$

$$\log k^C_N = \log k^C - \log E^{N-C} \quad (7)$$

$$\log k^C_{O,N} = \log k^C_0 - \log E^{N-C} \quad (8)$$

Applying Equations 1 and 3 for methyl 3-nitrotyrosinate:

$$K_1 = k^C_0 + k^N_0 \quad (9)$$

$$\log K_1 + \log K_2 = \log k^O_0 + \log k^C_0 \quad (10)$$

Furthermore, $\log k^N_{C,O}$ can be expressed from Equation 4:

$$\log k^N_{C,O} = \log k^N_N - \log E^{O-N} \quad (11)$$

Then, $\log k^N_{C,O}$ was introduced into Equation 10:

$$\log K_1 + \log K_2 = \log k^O_0 + \log k^N_C - \log E^{O-N} \quad (12)$$

Equations 9 and 12 constitute a system of equations with two unknown parameters ($\log k^C_0$ and $\log k^N_0$), resulting in values of 7.06 and 7.41 in log units. Equations 9 and 12, however, do not contain information on the assignments, i.e., which of the above values belong to $\log k^C_0$ and $\log k^N_0$. The chemical shift-pH profiles (Figure 3), chemical evidences and microconstant values in Table 3 unequivocally indicate that insertion of the electron-withdrawing nitro group in the aromatic ring reduces the basicity of the phenolate to a much greater extent than that of the amino.

Taking also the low diversity of the $\log k^N_0$ values into account, the two parameters could be identified, which also afforded the calculation of the remaining six protonation microconstants using the pair-interactivity parameters and the appropriate form of Equation 4:

Table 3. The $\log k^O_C$ and $\log k^N_C$ microconstants of tyrosine, 3-iodotyrosine and diiodotyrosine from ref. [7].

| Parameter | Tyrosine | 3-Iodotyrosine | Diiodotyrosine |
|-----------|----------|----------------|----------------|
| $\log k^O_C$ | 9.76     | 8.42           | 6.54           |
| $\log k^N_C$ | 7.58     | 7.53           | 7.41           |
After all the microscopic protonation constants of 3-NT were determined, some of the values could be verified by the self-consistence of the system. The value of $K_1$ can be calculated using Equation 1; additionally, $K_3 \approx k_{CN}^C$, since the protonation along the $k_{CN}^C$ arrow overwhelmingly predominates over the $k_{CN}^O$ and $k_{CO}^C$. The results of both checking procedure were within the margin of error. All 12 microconstants are shown in Table 4.

The pH-dependent distribution of the eight microspecies (Figure 4) was calculated based on the protonation microconstants in Table 4.

Comparing the microconstants of Tyr, 3-iodotyrosine and 3-NT, it is found that the basicity of the carboxylate and amino sites are similar, unlike the phenolate. This is straightforward to interpret, considering how distant the iodo or nitro group is from the protonation sites. The microconstants describing the phenolate basicity in three compounds are collected Table 4.

| Phenolate microconstants | Amino microconstants | Carboxylate microconstants |
|--------------------------|----------------------|---------------------------|
| $\log k_O^0$            | $\log k_O^N$         | $\log k_C^C$              |
| 7.35 ± 0.05             | 9.45 ± 0.05          | 4.53 ± 0.01               |
| $\log k_O^N$            | $\log k_O^C$         |                           |
| 6.82 ± 0.06             | 8.92 ± 0.05          | 4.24 ± 0.05               |
| $\log k_C^O$            | $\log k_C^N$         |                           |
| 7.06 ± 0.07             | 7.41 ± 0.03          | 2.49 ± 0.04               |
| $\log k_C^{NO}$         | $\log k_C^{CN}$      |                           |
| 6.53 ± 0.04             | 6.88 ± 0.05          | 2.20 ± 0.07               |

Figure 4. Logarithmic distribution diagram for all the microspecies of 3-nitrotyrosine.
Table 5. The phenolate microconstants of tyrosine, 3-iodotyrosine and 3-nitrotyrosine.

|                      | Tyrosine | 3-Iodotyrosine | 3-Nitrotyrosine |
|----------------------|----------|----------------|-----------------|
| $\log k_0^O$         | 10.04    | 8.71           | 7.35            |
| $\log k_N^O$         | 9.65     | 8.18           | 6.82            |
| $\log k_C^O$         | 9.76     | 8.42           | 7.06            |
| $\log k_{CN}^O$      | 9.37     | 7.89           | 6.53            |

in Table 5 (while all of the microconstants in Tyr, 3-iodotyrosine and 3-NT were summarized in the Supporting Information, Table S1).

The substitution of tyrosine with iodine decreases all the respective phenolate microconstants by some 1.4 log units. Changing the iodo group to nitro group results in greater changes and even lower protonation constants. Compared to tyrosine, the decrease is around 2.7 log units. This phenomenon can be explained by the stronger electron withdrawing effect and the negative mesomeric effect of the nitro group. Due to the phenolate microconstants, 89% of the phenolic OH groups are deprotonated in 3-NT molecules at the pH of the blood plasma (7.40).

Conclusions

Proteins with 3-NT obtain an extra negative site at physiological pH, with several intra- and intermolecular consequences: the ‘new’ anionic site attracts cationic counterparts, such as arginine guanidinium, lysine, ornithine, terminal ammonium, histidine imidazolium moieties, typically present in the same peptide or protein molecule, causing inevitable conformational modifications. Also, the anionic phenolate is prone to act as an anchor unit to associate with other biomolecules of cationic loci, and to bind metal ions. These changes in conformation, association and composition may all bring about alterations in the biological behavior.\(^{[3,4,10,11]}\)

Experimental Section

Materials

3-Nitro-L-tyrosine ((2S)-2-amino-3-(4-hydroxy-3-nitrophenyl)propanoic acid), 3-nitrotyrosine methyl ester (methyl (2S)-2-amino-3-(4-hydroxyphenyl)propanoate) and the internal NMR-pH indicators (imidazole, sarcosine, acetone oxime, sodium acetate) were obtained from Sigma-Aldrich. Deuteron oxide (D\(_2\)O) and methanol were purchased from Merck. All reagents were of analytical grade, obtained from commercial suppliers. The deionized water was prepared with a Milli-Q Direct 8 Millipore Water Purification System.

NMR Spectroscopy Measurements

All NMR measurements were carried out on a Varian 600 MHz spectrometer with a dual 5 mm inverse-detection gradient probe head at 25°C. The NMR-pH titrations were performed in a mixture of 5% D\(_2\)O/95% H\(_2\)O (v/v). For titration at 0.15 m ionic strength acidic and basic stock solutions were prepared with concentrations of 0.1 m HCl/NaOH and 0.05 m KCl to ensure 0.15 m ionic strength. The spectra were referenced to internal DSS (sodium 3-(trimethylsilyl)-1-propanesulfonate). The sample volume was 600 μl, the titrant and pH indicator concentrations were 5 mm. In the \(^1\)H-NMR experiments, pH values were determined by internal indicator molecules optimized for NMR.\(^{[12,13]}\) The concentration of the analytes was 1 mm for the titration. The water resonance was diminished by a double pulse field gradient spin echo sequence (number of transients = 16, number of points = 16384, acquisition time = 3.33 s, relaxation delay = 1.5 s).

Data Analysis

For the analysis of NMR titration curves of proton chemical shifts versus pH, the software Origin Pro 8 (OriginLab Corp., Northampton, MA, USA) was used. In \(^1\)H-NMR-pH titrations the non-linear curve fitting regression analysis option was used with the following function:\(^{[8]}\)

$$\delta_{\text{abs}(\text{pH})} = \delta_{L^2-} + \sum_{i=0}^{n-1} \delta_{L^{2-}2^{i}} \cdot 10^{(\log b_{i+1} - \text{pH})}$$  

(19)

where $\delta_{L^2-}$ is the chemical shift of the unprotonated ligand ($L^2-$), $\delta_{L^{2-}2^{i}}$ values stand for the chemical shifts of successively protonated species of $L^2-$, $n$ is the maximum number of protons that can bind to the unprotonated ligand, $\beta$ is the cumulative protonation macroconstant. The standard deviations of log$\beta$ values from the regression analyses were used to calculate the Gaussian propagation of uncertainty for the other equilibrium constants.
Acknowledgements

This article was published under the frame of Semmelweis University, Faculty of Pharmacy and Transylvanian Museum Society, project No. 20.4/2018/P.2/EMEOGYSZ. The financial support from Semmelweis Innovation Found STIA–M-17and STIA_18_KF are highly appreciated. This work was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (G. Tóth).

Author Contribution Statement

G. Tóth and B. Noszál designed the study. T. Pálla and E. Fogarasi performed the NMR-pH titration. T. Pálla evaluated the data. All authors contributed equally to draft and revise the manuscript.

References

[1] S. Bartesaghi, R. Radi, ‘Fundamentals on the biochemistry of peroxynitrite and protein tyrosine nitration’, Redox. Biol. 2018, 14, 618–625.
[2] M. D. Croitoru, F. Ibolya, M. C. Pop, T. Dergez, B. Mitroi, M. T. Dogaru, B. Tőkés, ‘Nitrones are able to release nitric oxide in aqueous environment under hydroxyl free radical attack’, Nitric Oxide 2011, 25, 309–315.
[3] C. Batthyány, S. Bartesaghi, M. Mastrogiovanni, A. Lima, V. Demicheli, R. Radi, ‘Tyrosine-nitrated proteins: Proteomic and bioanalytical aspects’, Antioxid. Redox Signaling 2017, 26, 313–328.
[4] V. De Filippis, R. Frasson, A. Fontana, ‘3-Nitrotyrosine as a spectroscopic probe for investigating protein–protein interactions’, Protein Sci. 2006, 15, 976–986.
[5] P. M. Dewick, ‘Essentials of organic chemistry: for students of pharmacy, medicinal chemistry and biological chemistry’, John Wiley & Sons, 2006.
[6] H. Ren, L. Wang, X. Wang, X. Liu, S. Jiang, ‘Measurement of acid dissociation constants and ionic mobilities of 3-nitrotyrosine and 3-chloro-tyrosine by capillary zone electrophoresis’, J. Pharm. Biomed. Anal. 2013, 77, 83–87.
[7] G. Toth, S. Hosztafi, Z. Kovacs, B. Noszal, ‘The site-specific basicity of thyroid hormones and their precursors as regulators of their biological functions’, J. Pharm. Biomed. Anal. 2012, 61, 156–164.
[8] Z. Szakács, B. Noszál, ‘Protonation microequilibrium treatment of polybasic compounds with any possible symmetry’, J. Math. Chem. 1999, 26, 139–155.
[9] R. B. Martin, J. T. Edsall, D. B. Wetlauffer, B. R. Hollingworth, ‘A complete ionization scheme for tyrosine, and the ionization constants of some tyrosine derivatives’, J. Biol. Chem. 1958, 233, 1429–1435.
[10] R. Radi, ‘Protein tyrosine nitration: biochemical mechanisms and structural basis of functional effects’, Acc. Chem. Res. 2012, 46, 550–559.
[11] M. Tien, B. S. Berlett, R. L. Levine, P. B. Chock, E. R. Stadtman, ‘Peroxynitrite-mediated modification of proteins at physiological carbon dioxide concentration: pH dependence of carbonyl formation, tyrosine nitration, and methionine oxidation’, Proc. Natl. Acad. Sci. USA 1999, 96, 7809–7814.
[12] G. Orgován, B. Noszál, ‘Electrodeless, accurate pH determination in highly basic media using a new set of 1H-NMR pH indicators’, J. Pharm. Biomed. Anal. 2011, 54, 958–964.
[13] Z. Szakács, G. Hägele, R. Tyka, ‘1H/31P NMR pH indicator series to eliminate the glass electrode in NMR spectroscopic pKa determinations’, Anal. Chim. Acta 2004, 522, 247–258.

Received June 28, 2019
Accepted July 30, 2019