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Original Contribution

The kinetics of the reaction of nitrogen dioxide with iron(II)- and iron(III) cytochrome *c*

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ABSTRACT

The reactions of $NO_2^{\bullet}$ with both oxidized and reduced cytochrome *c* at pH 7.2 and 7.4, respectively, and with *N*-acetyltyrosine amide and *N*-acetyltryptophan amide at pH 7.3 were studied by pulse radiolysis at 23 °C. $NO_2^{\bullet}$ oxidizes *N*-acetyltyrosine amide and *N*-acetyltryptophan amide with rate constants of $(3.1 \pm 0.3) \times 10^5$ and $(1.1 \pm 0.1) \times 10^6\ M^{-1}\ s^{-1}$, respectively. With iron(III)cytochrome *c*, the reaction involves only its amino acids, because no changes in the visible spectrum of cytochrome *c* are observed. The second-order rate constant is $(5.8 \pm 0.7) \times 10^6\ M^{-1}\ s^{-1}$ at pH 7.2. $NO_2^{\bullet}$ oxidizes iron(II)cytochrome *c* with a second-order rate constant of $(6.6 \pm 0.5) \times 10^7\ M^{-1}\ s^{-1}$ at pH 7.4; formation of iron(III) cytochrome *c* is quantitative. Based on these rate constants, we propose that the reaction with iron(II) cytochrome *c* proceeds via a mechanism in which 90% of $NO_2^{\bullet}$ oxidizes the iron center directly—most probably via reaction at the solvent-accessible heme edge—whereas 10% oxidizes the amino acid residues to the corresponding radicals, which, in turn, oxidize iron(II). Iron(II)cytochrome *c* is also oxidized by peroxynitrite in the presence of CO_2 to iron(III)cytochrome *c*, with a yield of ~60% relative to peroxynitrite. Our results indicate that, in vivo, $NO_2^{\bullet}$ will attack preferentially the reduced form of cytochrome *c;* protein damage is expected to be marginal, the consequence of formation of amino acid radicals on iron(III)cytochrome *c*.

Cytochrome *c* is a small heme protein that has two physiological roles. It transfers electrons from cytochrome c_1 to cytochrome *c* oxidase in the respiratory chain and serves as a cytoplasmic apoptosis-triggering agent. Cytochrome *c* reacts with various inorganic and organic radicals [1], with inorganic complexes [2], and with a number of biologically important molecules, such as ascorbate [3] and peroxynitrite [4]. Electron transfer takes place at the solvent-accessible heme edge; in the case of negatively charged oxidants and reductants the reaction is favored [2,5] by the evolutionarily conserved asymmetric charge distribution of cytochrome *c* [6,7]. The heme iron is not accessible to the solvent and, thus, participates in electron transfer by an outer-sphere mechanism.

Abbreviations: trivial terms (in italic) and systematic names for the compounds used in this work are $AA^{\bullet}$ amino acid radical; ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) diammonium salt; $ABTS^{2-}$, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate); $ABTS^{\bullet-}$, radical of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate); $Br_2^{\bullet-}$, *bromine radical,* dibromide ($^{\bullet}1-$); CO_2, *carbon dioxide,* dioxidocarbon; $CO_2^{\bullet-}$, *formyl radical anion,* dioxidocarbonate ($^{\bullet}1-$); $CO_3^{\bullet-}$, *carbonate radical,* trioxidocarbonate ($^{\bullet}1-$); e_{aq}^-, hydrated electron; GSH, glutathione; $H^{\bullet}$, hydrogen atom; H_2O_2, *hydrogen peroxide,* dioxidane; Fe(III)cyt *c*, iron(III)cytochrome *c*; Fe(II)cyt *c*, iron(II)cytochrome *c*; KH_2PO_4, *potassium dihydrogen phosphate,* potassium dihydroxidodioxidophosphate; K_2HPO_4, *dipotassium hydrogen phosphate,* dipotassium hydroxidotrioxidophosphate; $K_3Fe(CN)_6$, *potassium ferricyanide,* tripotassium hexacyanidoferrate; NaH_2PO_4, *sodium dihydrogen phosphate,* sodium dihydroxidodioxidophosphate; Na_2HPO_4, *disodium hydrogen phosphate,* disodium hydroxidotrioxidophosphate; $NaNO_2$, *sodium nitrite,* sodium dioxidonitrate; *N*-acetyltryptophan amide, *N*-ac-Trp-NH_2 and *N*-ac-TrpH-NH_2; *N*-acetyltyrosine amide, *N*-ac-Tyr-NH_2 and *N*-ac-TyrOH-NH_2; *N*-ac-Trp$^{\bullet}$-NH_2, *N*-acetyltryptophan amide radical; *N*-ac-TyrO$^{\bullet}$-NH_2, *N*-acetyltyrosine amide radical; $N_3^{\bullet}$, *azide radical,* trinitrogen ($^{\bullet}$); N_2O, *nitrous oxide,* dinitrogen monoxide, oxidodinitrogen; $NO^{\bullet}$, *nitric oxide,* nitrogen monoxide, oxidonitrogen ($^{\bullet}$); $NO_2^{\bullet}$, *nitrogen dioxide,* dioxidonitrogen ($^{\bullet}$); NO_2^-, *nitrite,* dioxidonitrate (1−); $NO_2^{\bullet}2-$, *hydronitrite radical anion,* dioxidonitrate ($^{\bullet}2-$); $O_2^{\bullet}-$, *superoxide,* dioxide ($^{\bullet}1-$); $ONOO^-$, *peroxynitrite,* (dioxido)oxidonitrate (1−); ONOOH, *peroxynitrous acid,* (hydridodioxido)oxidonitrogen; $(SCN)_2^{\bullet}-$, *thiocyanate radical,* bis(nitridosulfidocarbonate) (S–S) ($^{\bullet}1-$); TyrO$^{\bullet}$, tyrosine radical.

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It has been shown that Tyr residues in cytochrome *c* are nitrated when exposed to peroxynitrite [8] or in the presence of H_2O_2 and NO_2^- via a peroxidase-like mechanism [9,10]. Nitrated cytochrome *c* is unable to support electron transfer in the respiratory chain [8,11]. Very recently, Silkstone et al. [12] postulated that $NO_2^{\bullet}$—formed during the autoxidation of $NO^{\bullet}$ in vitro—oxidizes iron(II)cytochrome *c* to iron(III)cytochrome *c;* they were able to fit their experimental results by a kinetic model assuming a rate constant of $3 \times 10^6\ M^{-1}\ s^{-1}$ for this reaction.

$NO_2^{\bullet}$ has a standard electrode potential, $E^o(NO_2^{\bullet}/NO_2^-)$, of 1.04 V vs NHE [13] and is expected to participate in many pathophysiological processes [14,15]. The principal pathway for the formation of $NO_2^{\bullet}$ in vivo is the reaction of peroxynitrite ($ONOO^-$/ONOOH, $pK_a = 6.8$) with CO_2 [16–20]. Other potential endogenous reactions that lead to the formation of $NO_2^{\bullet}$ are oxidation of NO_2^- by heme proteins [21,22] and in particular by proteins with peroxidase activity [23]. In vivo, formation of $NO_2^{\bullet}$ by $NO^{\bullet}$ autoxidation is negligible in aqueous domains [24]. $NO_2^{\bullet}$ can react with various compounds via addition to double bonds [25,26], hydrogen abstraction [27], or electron transfer mechanisms [28]. It oxidizes Tyr, Trp, and Cys with moderate rates, a number of proteins, and the anions of the unsaturated fatty acids linoleic and arachidonic acid [29,30]. We have shown that $NO_2^{\bullet}$ oxidizes iron in nitrosyl(II) hemoglobin and nitrosyl(II)myoglobin [31]. $NO_2^{\bullet}$ also rapidly recombines with other radicals. The very fast reaction of $NO_2^{\bullet}$ with $O_2^{\bullet -}$ leads to the formation of the strong oxidizing agent peroxynitrate [32], whereas reaction of $NO_2^{\bullet}$ with tyrosine, $TyrO^{\bullet}$, or tryptophan, $Trp^{\bullet}$, radicals in peptides or proteins produces nitrotyrosine or nitrotryptophan, respectively [30,33]. Nitrated proteins have been detected in vivo under many pathological conditions [14].

$ONOO^-$ reacts rapidly with CO_2 and forms to a limited extent $NO_2^{\bullet}$ and $CO_3^{\bullet -}$. We have already reported that $CO_3^{\bullet -}$ reacts with both iron(III)- and iron(II)cytochrome *c* with rate constants of 5.1×10^7 and $1.1 \times 10^9\ M^{-1}\ s^{-1}$, respectively [34]. It reacts both with the amino acid residues of iron(III)cytochrome *c* and with the exposed edge of the heme in iron(II)cytochrome *c*. The latter reaction results in a quantitative oxidation of the iron(II).

Here we report on the reaction of $NO_2^{\bullet}$ with both the reduced and the oxidized form of cytochrome *c* and further with *N*-ac-Tyr-NH_2 and *N*-ac-Trp-NH_2. In particular, we measured the corresponding rate constants and we investigated whether the heme or the protein moiety is preferentially attacked.

Experimental procedures

Materials

Cytochrome *c* from horse heart (96%, not prepared with trichloroacetic acid) and ABTS were supplied from Sigma and *N*-ac-Tyr-NH_2 and *N*-ac-Trp-NH_2 were from Bachem. $NaNO_2$ (99.999% on a trace metal basis) was from Aldrich Fine Chemicals or from Sigma, NaH_2PO_4 and Na_2HPO_4 (99.99%) were from POCH SA, and KH_2PO_4 and K_2HPO_4 (Suprapur 99.99%) were from Merck. The ascorbic acid (> 99.9999) and $NaNO_3$ (puriss. p.a.) were from Fluka, and $K_3Fe(CN)_6$ (puriss. p.a.) was from Sigma–Aldrich. We synthesized peroxynitrite by the method of Bohle et al. [35]. Water was purified with a Millipore Milli-Q unit.

Cytochrome c solutions

Stock solutions of iron(III)- or iron(II)cytochrome *c* in 10 mM sodium or 0.10 M potassium phosphate buffer and pH 7.2 or 7.4 were prepared by oxidizing or reducing cytochrome *c* with $K_3Fe(CN)_6$ and ascorbate, respectively, and passing the solution through a Sephadex-G-25 column. The solutions were kept on ice and used the same day.

The iron(III)cytochrome *c* solutions used for the pulse radiolysis experiments were prepared by adding appropriate amounts of deaerated stock solutions to 10 mM $NaNO_2$ solutions in 10 mM phosphate buffer, pH 7.2, saturated with N_2O. For iron(II)cytochrome *c* slightly different conditions were used: 5 mM $NaNO_2$, 0.10 M phosphate buffer, pH 7.4.

The concentration of iron(III)cytochrome *c* was determined spectrophotometrically at 410 nm, at which its molar absorption coefficient (ε) is $1.06 \times 10^5\ M^{-1}\ cm^{-1}$ [36,37], whereas that of iron(II)cytochrome *c* was determined at 550 nm from the absorbance difference between reduced and oxidized cytochrome *c* with $\Delta\varepsilon = 21.1 \times 10^3\ M^{-1}\ cm^{-1}$ [38].

Pulse radiolysis

For iron(III)cytochrome *c,* pulse radiolysis experiments were performed with a 6-MeV linear accelerator at the Gray Cancer Institute (GCI; Oxford, UK; 0.2-μs pulses, dose per pulse 2.0–2.2 Gy, optical path cell 2 cm) and at the Institute of Applied Radiation Chemistry (IARC; Lodz University of Technology, Lodz, Poland; 3-ns pulses, dose per pulse 6–8 Gy, optical path cell 1 cm). The spectrophotometric detection system used a xenon arc lamp as a light source, monochromators Spex Minimate and Mono Spectra Pro 275, Hamamatsu photomultipliers R277 and R928, home-made amplifiers, and digital storage Tektronix oscilloscopes DSA602 and TDS540, at the GCI and IARC, respectively [39,40]. For iron(II)cytochrome *c,* *N*-ac-Tyr-NH_2, and *N*-ac-Trp-NH_2, a 2.3-MeV Febetron 705 electron accelerator (Titan Systems Corp.) at the Institute of Inorganic Chemistry (Swiss Federal Institute of Technology, Zurich, Switzerland; 50-μs pulse width, dose per pulse applied 3–10 Gy) was used. The light source was a 75-W xenon arc lamp and the optical path length of the quartz cell (Hellma GmbH & Co., Jena, Germany) was 1 or 2 cm. The detection system consisted of an Acton SP300 monochromator (Roper Scientific) and a R928 photomultiplier (Hamamatsu), a DHPCA200 amplifier (Femto Messtechnik GmbH), and a DL7100 digital storage oscilloscope (Yokogawa Electric Corp.). The dose per pulse was determined with the thiocyanate dosimeter [41]. All experiments were carried out at 23 °C. Results were analyzed with Origin 6.0 (OriginLab) or Kaleidagraph from Synergy Software (Reading, PA, USA) or simulated with the Gen-int5 simulator (EXCEL-Macro). Error bars in the figures represent standard deviations (*s*) from the mean value. Second-order rate constants are given as (mean value $\pm\ t_s s/n^{0.5}$) for 95% probability.

Generation of $NO_2^{\bullet}$

For the reaction with cytochrome *c,* $NO_2^{\bullet}$ was generated upon irradiation of aqueous solutions of $NaNO_2$ (10 or 5 mM) in phosphate buffer (10 mM or 0.10 M), pH 7.2 or 7.4, which contained 0–93 μM cytochrome *c* and were saturated with N_2O (24.4 mM). During water radiolysis, e_{aq}^-, $HO^{\bullet}$, and $H^{\bullet}$ are generated with yields of 0.28, 0.28, and 0.06 μmol J^{-1} [42]. Under the experimental conditions used for the oxidized and reduced forms of cytochrome *c,* about 85 and 92% of e_{aq}^-, respectively, is converted to $HO^{\bullet}$ [43] (Reaction (1)), and this, in turn, to $NO_2^{\bullet}$ [32] (Reaction (2)):

$$e_{aq}^- + N_2O + H_2O \rightarrow HO^{\bullet} + N_2 + OH^-, \quad (1)$$

$$HO^{\bullet} + NO_2^- \rightarrow OH^- + NO_2^{\bullet}. \quad (2)$$

The rest of the e_{aq}^- is expected to react mainly with NO_2^- to give $NO_2^{\bullet 2-}$ [44,45] and finally $NO^{\bullet}$ [46]; less than 1.5% of the e_{aq}^- would react with the protein [47,48]. $HO^{\bullet}$ and $H^{\bullet}$ react with iron(III) cytochrome *c* with rate constants of 1.4×10^{10} [49] and $1.1 \times 10^{10}\ M^{-1}\ s^{-1}$ [50,51] to give, among other products, 25–55 [52] and 50% iron(II)cytochrome *c* [50,51], respectively. The corresponding