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Original Contribution

Peroxynitrite-derived carbonate and nitrogen dioxide radicals readily react with lipoic and dihydrolipoic acid

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Abstract

α-Lipoic acid (LA) and dihydrolipoic acid (DHLA) may have a role as antioxidants against nitric oxide-derived oxidants. We previously reported that peroxynitrite reacts with LA and DHLA with second-order rate constants of 1400 and 500 M^{-1} s^{-1}, respectively, but indicated that these direct reactions are not fast enough to protect against peroxynitrite-mediated damage in vivo. Moreover, the mechanism of the reaction of peroxynitrite with LA has been recently challenged (*J. Biol. Chem.* **279**:9693–9697; 2004). Pulse radiolysis studies indicate that LA and DHLA react with peroxynitrite-derived nitrogen dioxide ($^{\bullet}NO_2$) ($k_2 = 1.3 \times 10^6$ and 2.9×10^7 M^{-1} s^{-1}, respectively) and carbonate radicals ($CO_3^{\bullet-}$) ($k_2 = 1.6 \times 10^9$ and 1.7×10^8 M^{-1} s^{-1}, respectively). Carbonate radical-mediated oxidation of LA led to the formation of the potent one-electron oxidant LA radical cation. LA inhibited peroxynitrite-mediated nitration of tyrosine and of a hydrophobic tyrosine analog, *N-t*-BOC L-tyrosine *tert*-butyl ester (BTBE), incorporated into liposomes but enhanced tyrosine dimerization. Moreover, while LA competitively inhibited the direct oxidation of glutathione by peroxynitrite, it was poorly effective against the radical-mediated thiol oxidation. The mechanisms of reaction defined herein allow to rationalize the biochemistry of peroxynitrite based on direct and free radical-mediated processes and contribute to the understanding of the antioxidant actions of LA and DHLA.

Keywords: Peroxynitrite; Lipoic acid; Carbonate radical; Nitrogen dioxide; Dihydrolipoic acid; BTBE

Introduction

Peroxynitrite[1] is a powerful oxidant formed in vivo by the diffusion-controlled reaction between nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$) radicals and contributes as a pathogenic mediator in a variety of diseases states [1–4]. Peroxynitrous acid ($pK_a = 6.8$) decays in a first order process ($k = 0.9$ s^{-1} at

Abbreviations: LA, α-Lipoic acid; DHLA, dihydrolipoic acid; DTPA, diethylenetriaminepentaacetic acid; DTNB, 5,5′-dithio-2-nitrobenzoic acid; BTBE, *N-t*-BOC L-tyrosine *tert*-butyl ester; 3-nitroBTBE, 3-nitro *N-t*-BOC L-tyrosine *tert*-butyl ester; DMPC, 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine.

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[1] IUPAC recommended names for peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH) are oxoperoxonitrate (1-) and hydrogen oxoperoxonitrate, respectively. The term peroxynitrite is used to refer to the sum of $ONOO^-$ and ONOOH.

pH 7.4 and 37°C) yielding nitrate, through homolysis of the peroxidic oxygens yielding hydroxyl (•OH) and nitrogen dioxide (•NO_2) radicals [5,6] in approximately 30% yields [7,8]. In biological systems, transition metal centers and thiols constitute main targets for peroxynitrite [9]. Moreover, the rapid reaction of peroxynitrite anion with CO_2 ($k_2 = 5.8 \times 10^4$ M^{-1} s^{-1} at 37°C [10,11]) results in carbonate radical anion ($CO_3^{\bullet-}$) and nitrogen dioxide (•NO_2) in about 35% yields [12–14]. The one-electron oxidants $CO_3^{\bullet-}$ and •NO_2 ($E^{\circ\prime}_{CO_3^{\bullet-}/CO_3^{2-}}$ = 1.78 V [15,16]; $E^{\circ\prime}_{\bullet NO_2/NO_2^-}$ = 0.99 V [17]) can in turn mediate several peroxynitrite-dependent oxidations and nitrations [15,18–20].

Since the initial proposal of the biological formation and reactions of peroxynitrite, a series of authors suggested through the years the existence of alternative conformational states of peroxynitrite to account for some of the reaction chemistries observed. While possible conformations of peroxynitrite have been anticipated based on quantum calculations and spectral data [9], the rate of interconversion in aqueous solutions is very fast [21] and therefore their relevance on peroxynitrite biochemistry is questionable and unlikely. The postulated species include *cis* and *trans* conformers of peroxynitrite anion and peroxynitrous acid [17,22,23] and a "bipolar" conformation of peroxynitrite in which internal electron density distribution creates a "nitronium ion-like" moiety [24,25]. However, the accumulation of experimental data and computer-assisted simulations indicate that there is no need to invoke these conformers to explain the reactivity of peroxynitrite with biological targets, and in most, if not all, cases, can be rationalized on the basis of either direct bimolecular reactions or through the reactions of peroxynitrite-derived radical intermediates, namely •OH, and •NO_2, and if carbon dioxide is present, $CO_3^{\bullet-}$ [9,14,26–28].

The disulfide α-lipoic acid (LA)[2] and its reduced form, dihydrolipoic acid (DHLA)[2], play a central role in cellular redox metabolism and may also serve an antioxidant function [31]. Indeed, dietary supplementation with LA has been successfully utilized in different oxidative stress models including diabetes [32,33]. A recent metaanalysis provided evidence that LA supplementation significantly improves both positive neuropathic symptoms and deficits to a clinically meaningful degree in diabetic patients with symptomatic polyneuropathy [34]. Dietary bioavailability studies show that an oral LA dose is rapidly absorbed and appreciably increases plasma LA levels, that can reach 0.2 μM concentrations [35], while in tissues can approach micromolar levels [36]. As LA and DHLA are both water- and fat-soluble antioxidants [37], they are considered to play a significant protective role against reactive oxygen species such as singlet oxygen [38], peroxyl radicals, and hypochlorite anions [31,39] both in aqueous and hydrophobic compartments.

We have previously shown that peroxynitrous acid reacts with LA with a second-order rate constant of 1400 M^{-1} s^{-1} at pH 7.4 and 37°C, yielding nitrite and LA-thiosulfinate (also known as β-lipoic acid) as reaction products [40]. The dithiol DHLA reacted with peroxynitrite with a relatively small second-order rate constant of 500 M^{-1} s^{-1} [40], in agreement with the high pK_a of its thiol groups (pK_a = 10.7) [40,41]. In a recent paper by Rezk et al. [25], the authors compared the ability of LA and glutathione (GSH) to protect against several peroxynitrite-mediated oxidations. The detection systems used were dihydrorhodamine-123 oxidation, α_1-antiproteinase inactivation, glutathione *S*-transferase P1-1 inactivation, and tyrosine nitration. The authors found that, despite the fact that LA and GSH react with peroxynitrite similarly fast (k_{LA} = 1400 vs k_{GSH} = 1350 M^{-1} s^{-1} [17,40]), their relative capacity to protect against peroxynitrite-mediated oxidations was rather different depending on the detector used. These results, together with what it was considered a low yield of peroxynitrite-dependent LA oxidation, prompted them to postulate a mechanism of reaction where only a fraction of peroxynitrite, which would be present in a bipolar conformation, could react with LA [25]. Moreover, the reaction of LA with the hypothetical bipolar conformation of peroxynitrite has been also recently invoked by Nakagawa et al. [42] in experiments involving peroxynitrite-dependent tyrosine oxidation: whereas LA inhibited tyrosine nitration, it *increased* tyrosine dimerization to 3,3′-dityrosine. The observations were ascribed to partially independent oxidative pathways that lead to either 3-nitrotyrosine (via the bipolar form) or 3,3′-dityrosine (via a radical pathway) [42]. However, it has been unambiguously demonstrated by ^{15}N chemically induced dynamic nuclear polarization and product yield analysis that a radical process is the main mechanism of peroxynitrite-mediated nitration of phenolics [43].

The proposition of alternative conformational states of peroxynitrite to account for its reactivity with LA as proposed by Rezk et al. [25] and Nakagawa et al. [42] is highly speculative. In the case of Rezk et al. [25] the data were obtained without appreciating that the detectors used have different mechanisms of peroxynitrite-mediated oxidation,[3] and that LA oxidation yields were not low, but the

[2] As the pK_a of the carboxylic acid moiety of LA and DHLA are ~4.8 [29,30], they exist mainly in the dissociated form (i.e., lipoate and dihydrolipoate) at physiological pH.

[3] The mechanism of peroxynitrite-mediated dihydrorhodamine oxidation was recently investigated by Glebska and Koppenol, 2003.The authors concluded that peroxynitrite does not react directly with DHR [44]. Moreover, peroxynitrite-derived radicals •OH, •NO_2, and $CO_3^{\bullet-}$ oxidize dihydrorhodamine [45]. The mechanism of peroxynitrite-dependent α_1-antiproteinase inactivation involves the direct oxidation of a critical methionine in the enzyme [46]. Peroxynitrite-dependent glutathione transferase P1-1 inactivation relies on the nitration of a critical tyrosine [47]. Peroxynitrite-dependent tyrosine nitration is a radical process which depends on peroxynitrite homolysis to •OH and •NO_2 and subsequent radical-mediated tyrosine oxidation to tyrosyl radicals and recombination of tyrosyl radicals with •NO_2 [48].