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Antioxidant Reactions of Dihydrolipoic Acid and Lipoamide with Triplet Duroquinone

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The oxidation of the antioxidants dihydrolipoate and lipoamide by triplet duroquinone (^{3}DQ) has been studied by laser flash photolysis and time-resolved resonance Raman (TR^3) spectroscopy. Reaction of ^{3}DQ with lipoamide by electron transfer [$k(H_2O)/k(D_2O \sim 1$] was more rapid than with dihydrolipoate, in which a proton is also involved [$k(H_2O)/k(D_2O \sim 2$]. For dihydrolipoate at neutral pH the undeprotonated form was the major reactive species with $k \sim 10^9$ dm^3 mol^{-1} s^{-1}. At higher pH values the reaction of ionised (thiolate) forms was observed with $k \geq 4 \times 10^9$ dm^3 mol^{-1} s^{-1}. The electron transfer mechanism of reaction between ^{3}DQ and lipoamide was confirmed by TR^3 spectra in which formation of the durosemiquinone radical anion and lipoamide disulfide radical cation ($RSS^{+\bullet}$) was observed.

Key Words: **lipoamide; dihydrolipoate; radical; oxidation; Raman.**

Thiols are widely regarded as having antioxidant [1] and radioprotective [2] powers by virtue of their reducing properties. Both dihydrolipoic acid and lipoic acid and derivatives have been intensively investigated recently as antioxidants [3, 4], although in nature (dihydro)lipoamide functions mainly as a cofactor in the pyruvate dehydrogenase complex. Lipoic acid and dihydrolipoic acid have been shown to be antioxidants in model systems and to scavenge reactive oxygen species (ROS) such as singlet oxygen [5], hypochlorous acid and the trichloromethylperoxyl radical [6], and hydroxyl radicals [6, 7]. In addition lipoic acid is capable of chelating redox-active transition metal ions [6, 8].

Despite the extensive investigation of the antioxidant properties of lipoic acid and dihydrolipoate *in vitro* and *in vivo*, there are fewer physiochemical studies of reactions between both of these compounds and ROS. Pulse radiolysis has been used to study both the one-electron oxidation and reduction of dihydrolipoate and lipoate respectively to the lipoate radical anion ($RSSR^{-\bullet}$) [9]. Asmus and Bonifacic have undertaken extensive studies showing that disulfides are oxidised to a radical cation ($RSSR^{+\bullet}$) [10] and have used pulse radiolysis to estimate the reduction potential of the lipoate radical cation $RSS^{+\bullet}$/RSS couple as 1.10 V vs SHE [11], some 300 mV lower than for that for non-cyclic disulfides and a result of the small dihedral CS-SC angle in the five-membered ring of lipoic acid [12]. The disulfide radical cation from lipoate is capable of oxidising the ionised forms of simple thiols with rate constants of the order of 10^9 dm^3 mol^{-1} s^{-1} [13]. The relative reduction potential of the radical from one-electron oxidation of dihydrolipoate has been determined by Surdhar and Armstrong, who note little difference in this regards between the dihydrolipoate radical and that from an open chain thiol such as β-mercaptoethanol at neutral pH [14].

Whilst it is usually accepted that the triplet excited state of duroquinone (^{3}DQ) is mainly π-π* in nature, its reactivity with ethanol and isopropanol [15, 16] implies at least partial n-π* character in polar solvents when the triplet may then be a model for peroxyl and alkoxyl radicals. Furthermore ^{3}DQ might be used to represent excited oxidant states that are formed in "dark" reactions in biochemical systems and which may be partially responsible for oxidative stress under some conditions [17]. We have previously studied the oxidation of a number of antioxidants by ^{3}DQ and have found it to react rapidly ($k_2 \sim 10^9$ dm^3 mol^{-1} s^{-1}) with dihydrolipoic acid in ethanol solution and to be a useful one-electron oxidant for the study of antioxidant radicals by time-resolved resonance Raman (TR^3) spectroscopy [18, 19].

MATERIALS AND METHODS

Dihydrolipoic acid was a gift from Dr. H. Tritschler (Asta Medica AG, Frankfurt, Germany). Other chemicals were obtained from Aldrich. Solutions were prepared using water obtained from a Milli Q apparatus and spectrophotometric grade acetonitrile.

Laser flash photolysis employed a Lumonics PM-846 XeCl laser emitting a 10 ns pulse at 308 nm. The laser output was attenuated

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to approximately 5-10 mJ pulse^{-1} in order to minimise triplet-triplet decay of excited duroquinone and all samples were deoxygenated by bubbling with argon. Transient absorption measurements were made using a conventional kinetic single beam spectrometer consisting of tungsten lamp (Oriel), monochromator (Bentham, model TM310) and photomultiplier detection (Hamamatsu R905). Time-resolved resonance Raman spectra were obtained using the apparatus previously described [18]. The pump pulse was the frequency-tripled output of a Continuum Sunlight Nd:YAG laser at 355 nm (5 ns, ~1 mJ) and the probe pulse was obtained from a Lumonics XeCl pumped dye laser (Lambda-Physik FL3002) at 440 nm(10 ns, ~0.5 mJ). Raman spectra were measured with a Spex Triplemate 1877 and a back-illuminated liquid nitrogen cooled CCD array (Princeton Instruments). Calibration was with the toluene Raman spectrum and is accurate to ±2 cm^{-1} and spectral manipulations were performed with the Princeton CSMA software.

RESULTS AND DISCUSSION

(a) Kinetics of triplet duroquinone reactions. When duroquinone is excited by the 308 nm output of the XeCl laser, the initial singlet excited state of duroquinone undergoes rapid (ca 20 ps) and efficient (ϕ = 0.91) intersystem crossing [20] to the triplet state which is a strong oxidant [21]. In an acetonitrile/water (1:1) solution ^{3}DQ (λ_{max} 480 nm) decays with a first order rate constant (k_0) of about 2 × 10^5 s^{-1}. In the presence of added reactive solute, S, (having a second order rate constant k_r) the pseudo first order decay (k′) of ^{3}DQ is accelerated and the value of k_r was determined from the slope of a plot of k′ versus [S] according to equation (1).

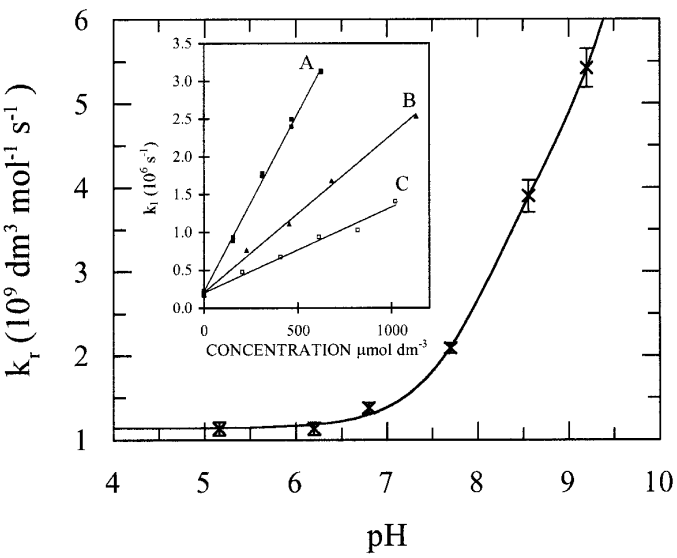
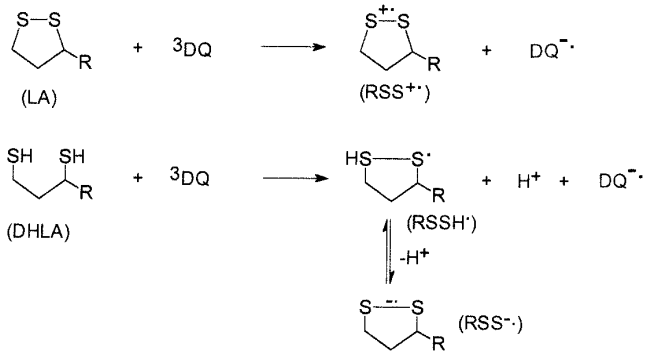


FIG. 1. Effect of pH on the second order rate constant, k_r, for reaction of triplet duroquinone with dihydrolipoate in phosphate (10 mmol dm^{-3}) buffered water/acetonitrile (50:50 v/v). (Inset) Plots of first order rate constants (k_1) for decay of the duroquinone triplet absorption at 480 nm versus concentration of lipoamide at pH 7.7 (A), dihydrolipoate at pH 7.7 (B), and dihydrolipoate at pH 6.2 (C). The dihydrolipoate solutions also contained EDTA (25 μmol dm^{-3}).



SCHEME 1.

$$k' = k_0 + k_r[S]. \quad [1]$$

The inset to Figure 1 shows such a plot (line B) for the oxidation of dihydrolipoate (DHLA) by ^{3}DQ at pH 7.7 from which a value for k_r of (2.09 ± 0.06) × 10^9 dm^3 mol^{-1} s^{-1} was obtained. During these measurements it was noted that on repeated pulse illumination of these solutions at higher laser intensity the rate of decay of ^{3}DQ increased, implying the formation of a more reactive product. This is confirmed by the plot in the inset to Figure 1 (line A) for the reaction of ^{3}DQ with lipoamide (LA) at pH 7.7, from which k_r = (4.72 ± 0.08) × 10^9 dm^3 mol^{-1} s^{-1}. Hence at pH 7.7 ^{3}DQ reacts more than twice as rapidly with LA as with DHLA. Below pH 6 the rate constant for reaction of ^{3}DQ with dihydrolipoate is independent of pH with a value of 1.1 × 10^9 dm^3 mol^{-1} s^{-1} but at pH values above 7 the rate constant increases indicating a much higher reactivity of the thiolate anion, as is observed for reaction of cysteine with free radicals [22] and singlet oxygen [23]. The results are consistent with the reported average pK_a of the thiol groups of DHLA of 10.7 [24]. The results in Figure 1 indicate that at pH 7, quenching of ^{3}DQ is due almost entirely to the undeprotonated form of DHLA. The unexpected results reported here showing that ^{3}DQ is more reactive with LA than with DHLA support the previous observation that the $CCl_3O_2^{\cdot}$ radical reacts nearly 8 times faster with lipoic acid than with DHLA [6].

Scheme 1 shows that the reaction of ^{3}DQ with LA is expected to be a simple electron transfer. Reaction of the DHLA in the undeprotonated form (pH ≪ pK_a ~ 10.7) involves both electron and proton loss to generate initially the monoprotonated radical (i.e., RSSH$^{\cdot}$) which may undergo further proton loss to give the cyclic disulfide radical anion (RSS$^{-\cdot}$). This is confirmed by the results of experiments in which D_2O replaced H_2O in the solvent. The k(H_2O)/k(D_2O) ratio for the reaction of ^{3}DQ with LA has a value (Table 1) of ~1 indicative of a simple electron transfer without the involvement of a proton or hydrogen atom. In contrast the reaction

TABLE 1

	Dihydrolipoic acid	Lipoamide
k_r, $dm^3\ mol^{-1}\ s^{-1}$	$(1.13 \pm 0.08) \times 10^9$ (pH 5.2)	$(4.72 \pm 0.08) \times 10^9$
$k(H_2O)/k(D_2O)$	1.97 ± 0.23	0.972 ± 0.021
Activation energy,[a] ΔE_a	4.2 ± 1.5	10.1 ± 0.7
Enthalpy of activation,[a] $\Delta H‡$	1.7 ± 1.5	7.6 ± 0.7
Entropy of activation,[a] $\Delta S‡$	-62 ± 2	-34 ± 1

[a] In units of kJ mol^{-1}.

^{3}DQ with DHLA at pH 5.2 (involving exclusively the undeprotonated form) has a $k(H_2O)/k(D_2O)$ ratio of ~2 clearly indicating the involvement of a proton in the reaction mechanism. The activation energies for reaction of ^{3}DQ with LA and DHLA are 10.1 ± 0.7 and 4.2 ± 1.5 kJ mol^{-1} respectively. These small values are expected for reactions whose rates approach the diffusion-controlled limit. The Table shows that the slower reaction of ^{3}DQ with DHLA, whilst having a lower experimental energy and enthalpy of activation, has a considerably more negative entropy of activation than the reaction of ^{3}DQ with LA. This entropic control of the slower reaction of ^{3}DQ with DHLA is to be anticipated for a reaction in which both a proton and electron are removed from the reductant, and in which the transition state may well include an ordered molecule of water.

(b) Transient absorption and resonance Raman spectra. Transient absorption spectra obtained by laser flash photolysis are shown in Figure 2. With both DHLA (Fig 2A) and LA (Fig 2B) the spectra obtained at pH 7.7 are consistent with one-electron oxidation of the substrate and show the decay of ^{3}DQ (λ_{max} 480 nm) and formation of the durosemiquinone radical anion, $DQ^{-\bullet}$ (λ_{max} 440 nm). In the reaction of ^{3}DQ with LA the $DQ^{-\bullet}$ radical yield is lower than in the case of DHLA. The product spectra from reaction of ^{3}DQ with DHLA are stable on the tens of microseconds timescale, whilst those from reaction with LA decay with a lifetime of approximately 15 ms. This is insufficient to account for the lower yield of the $DQ^{-\bullet}$ absorption and suggests that relatively efficient back electron transfer occurs from the geminate ($DQ^{-\bullet}$:$RSS^{+\bullet}$) radical ion pair. In the spectrum obtained at 5 ms after reaction of ^{3}DQ with LA (Figure 2B, curve d) there appears to be an additional component underlying the spectrum of $DQ^{-\bullet}$ ascribed to the lipoamide radical cation ($RSS^{+\bullet}$, Scheme 1) which has

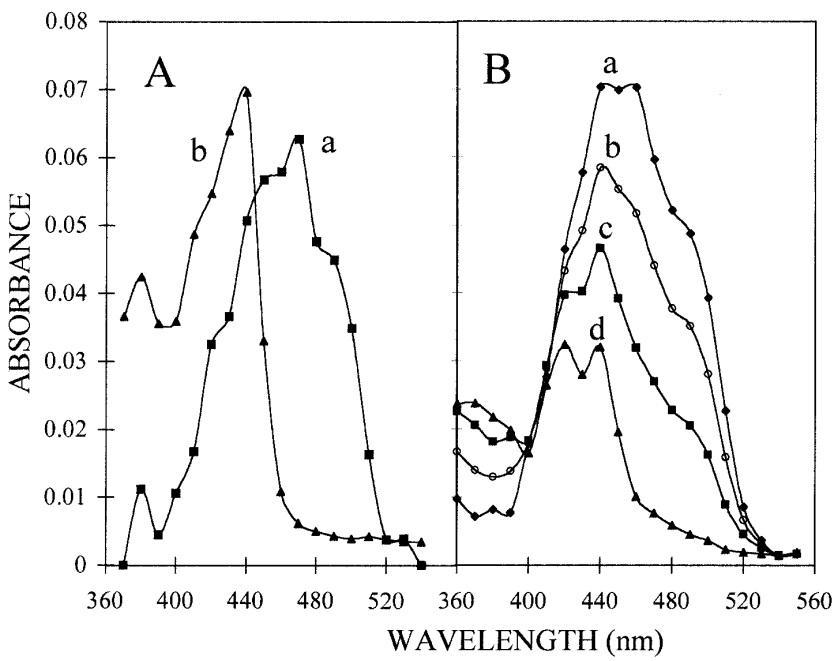


FIG. 2. Transient absorption from laser flash photolysis (308 nm) of DHLA and LA in deaerated solutions (acetonitrile/water, 50:50 v/v, 10 mmol dm^{-3} phosphate buffer, pH 7.7) of duroquinone (2 mmol dm^{-3}). (A) DHLA (0.4 mmol dm^{-3}) at 50 ns (a) and 10 ms (b) after the laser flash; (B) lipoamide (0.3 mmol dm^{-3}) at 50 ns (a), 300 ns (b), 800 ns (c), and 5 ms (d) after the laser flash.

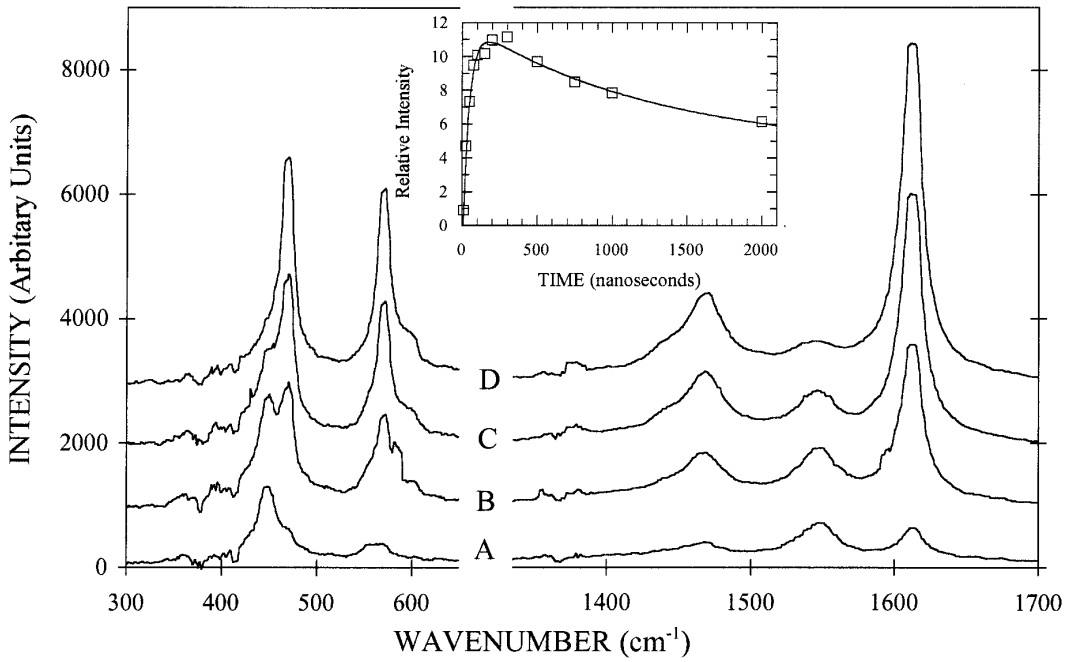


FIG. 3. Time-resolved resonance Raman spectra measured from acetonitrile/water (50:50 v/v) solutions of duroquinone (5 mmol dm^{-3}) with lipoamide (2 mmol dm^{-3}) buffered to pH 7.7 with phosphate (10 mmol dm^{-3}). Delays between the pump (355 nm) and probe (440 nm) laser pulses were 5 ns (A), 25 ns (B), 50 ns (C), and 100 ns (D). Background solvent spectra (measured using only the probe pulse) have been subtracted. (Inset) Intensity of the durosemiquinone radical anion peak at 1613 cm^{-1} as a function of delay time between pump and probe pulses.

a more intense and broader spectrum than $DQ^{-\bullet}$, but which is also centered at 440 nm [13].

Resonance Raman spectroscopy was also used to investigate the reaction between ^3DQ and LA. ^3DQ was formed by a 355 nm pump laser pulse and the Raman spectrum of reaction intermediates probed after a variable time delay by a second laser pulse at 440 nm, chosen to be in resonance with both the durosemiquinone radical anion and the lipoamide radical cation ($RSS^{+\bullet}$) absorption spectra. Figure 3 shows resonance Raman spectra in which the ^3DQ bands at 1548 cm^{-1} and 448 cm^{-1} decay with increasing time delay (from 5 to 100 ns) between pump and probe pulses and the concomitant formation of $DQ^{-\bullet}$ bands at 469, 570, 1470, and 1613 cm^{-1}. The latter two bands are assigned to the C—O and C=C stretching vibrations respectively of the durosemiquinone radical anion [18]. The time course of formation of the $DQ^{-\bullet}$ band at 1613 cm^{-1} (Inset to Figure 3) shows that the peak intensity is reached at 200 ns, and like the transient absorption spectra (Figure 2B) indicates that the products decay over a period of microseconds.

Inspection of the TR^3 spectrum in Figure 3 at a time delay of 100 ns (trace D) shows a shoulder on the higher wavenumber side of the 570 cm^{-1} band which cannot be ascribed to the durosemiquinone radical. In order to observe possible contributions from the LA disulfide radical cation, the TR^3 spectrum of $DQ^{-\bullet}$ (obtained from reaction of ^3DQ with sodium ascorbate under identical conditions - the ascorbate radical does not absorb at 440 nm and has not been observed by TR^3 [25]) was subtracted from the TR^3 spectrum from reaction of ^3DQ with lipoamide. The resulting expanded spectrum in Figure 4 reveals peaks at 298, 326, 598 and 1191 cm^{-1} and these are assigned to the lipoamide disulfide radical cation. Artefact peaks due to poor subtraction of solvent (acetonitrile) bands are indicated by an asterisk. The strongest peak in the lipoamide disulfide radical cation spectrum at 598 cm^{-1} is assigned to the S-S stretching mode, which in lipoic acid in methanol solution occurs at 504 cm^{-1} [26, 27]. The increase in vibrational frequency upon oxidation of the disulfide to the radical cation is consistent with the formation of the stronger three electron bond in the radical resulting from interaction of the singly occupied orbital on the sulphur atom representing the primary site of oxidation and an lone pair of electrons on the other sulphur atom [11, 12]. This contrasts with the situation in the disulfide radical anions such as $(SCN)_2^{-\bullet}$ in which the odd electron occupies a σ^* orbital causing considerable weakening of the S-S bond and a reduction in the vibrational frequency of the S—S bond from 490 cm^{-1} in $(SCN)_2^{-\bullet}$ to 218 cm^{-1} in $(SCN)_2^{-\bullet}$ [28]. There appear to be no discernible bands in Figure 4 attributable to the C—S stretches in the region of 700 $cm^{-1,}$ although in the Raman spectra of lipoic acid and lipoamide these

are less intense than the S—S band [26, 27]. The band at 1191 cm^{-1} is believed to be the first overtone of the S-S stretching vibration at 598 cm^{-1}. Series of overtones have been observed in the spectra of $(SCN)_2^{-\bullet}$ and dihalide radicals [28]. At present it is not possible to unambiguously assign the two bands at lower frequency (298 and 326 cm^{-1}) but we believe these to be either C—S bending modes or alkyl chain expansions pertaining to deformations of the C—C framework.

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