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Effect of ^{13}C-, ^{18}O- and ^{2}H-labeling on the infrared modes of UV-induced phenoxyl radicals

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Abstract

The structure and environment of redox active tyrosines present in several metalloenzymes can be studied by resonance Raman spectroscopy or Fourier transform infrared difference spectroscopy. Assignments of the vibrational modes in vivo often requires in vitro studies on model compounds. This approach is briefly reviewed. New results are shown on the influence of isotope-labeling on the infrared spectra of tyrosine, *p*-methylphenol and phenol radicals obtained in vitro by UV-irradiation. The infrared spectra of the radicals are dominated by the ν(CO) mode at 1515–1504 cm^{-1}. The frequency shifts induced on this mode by ^{13}C- ^{2}H-, and ^{18}O-labeling are reported. © 1998 Elsevier Science B.V.

Keywords: Infrared spectrum; Tyrosine; Radical; Isotope-labeling

1. Introduction

Tyrosine radicals ($Tyr^{\cdot}$) are present in the active site of many metalloenzymes including ribonucleotide reductase R2 [1,2], photosystem II (PSII) [3], prostaglandin synthase [4], bovine liver catalase [5], in a thioether modified form in galactose oxidase and glyoxal oxidase [6,7] and in a dihydroxylated form in copper amine oxidase [8]. $Tyr^{\cdot}$ have been proposed to initiate catalysis by proton and electron abstraction from substrate [9,10]. Tyrosyl radical properties may be tuned by specific interactions with the environment in the protein. These interactions and their consequences on the $Tyr^{\cdot}$ structure can be probed by vibrational spectroscopy. Such studies require comparisons with model spectra and the use of isotopically-labeled and/or ring substituted phenoxyl radicals. This is illustrated notably by the resonance Raman (RR) study of ribonucleotide re-

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Abbreviations: FTIR, Fourier transform infrared; RR, resonance Raman; PSII, photosystem II; *p*-Cresol, *p*-methylphenol; $^{2}H_4$-Tyr, tyrosine with fully deuterated ring; $^{13}C_6$-Tyr, tyrosine with the six ring carbons ^{13}C-labeled; $^{13}C_1(4)$-Tyr, tyrosine ^{13}C-labeled at the ring carbon C4 which binds the hydroxyl group; ν, Stretching vibration.

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ductase [11] and galactose oxidase [12], for which the identification of the Tyr$^{\cdot}$ vibrational modes was based on a large number of experimental works on phenoxyl radicals in vitro [12–19]. Phenoxyl or Tyr$^{\cdot}$ radicals are characterized by an intense mode at 1498–1515 cm^{-1} identified as the ν(CO) mode by RR using ^{17}O-labeling [19]. A second mode is observed by RR at 1550–1585 cm^{-1}, which is assigned to ring ν(CC) vibrations [12–19]. The frequency and intensity of this mode is sensitive to ring substitution and coordination of the phenoxyl radical to a metal [12,20–22].

For enzymes containing numerous pigments, study by RR is impaired. In these proteins, vibrational information can be obtained using Fourier transform infrared (FTIR) difference spectroscopy as illustrated by the study of Tyr$^{\cdot}$ formation in PSII [23–27]. To analyze the FTIR difference spectra, effect of isotope-labeling and ring substitutions on Tyr$^{\cdot}$ and phenoxyl radicals have been studied both in situ and in vitro [23–26,28]. Data in Refs. [26,27] however are in disagreement with those in Refs. [23–25]. The origin of this discrepancy, i.e., the misassignment of FTIR difference spectra of PSII in Refs. [26,27], has been discussed in detail elsewhere ([23], see also Refs. [29–31]).

Improvements in the calculation procedures relevant to the radical structures provide a range of predictions including the effects of ^{13}C-, ^{2}H-, ^{17}O-, ^{18}O-labeling, the effects of ring substitution, the effect of the radical protonation and the interaction with solvent (water) molecules [18,19,32–41].

In this work, we summarize the effects of ^{13}C-, ^{2}H-, and ^{18}O-labeling on the infrared modes of phenol$^{\cdot}$, *p*-cresol$^{\cdot}$, and Tyr$^{\cdot}$ obtained by UV-irradiation in solution.

2. Materials and methods

2.1. Tyrosine and p-cresol labeling

Tyrosine, *p*-cresol (*p*-methylphenol) and phenol of the best grade commercially available (from Aldrich or Sigma) were used. Phenol(d_6) (98% enrichment), was purchased from Aldrich. L-Tyrosine deuterated on the *ortho*-position of the OH group was prepared as described previously [28]. Ring $^{2}H_4$-labeled L-tyrosine (97%) was purchased from ISOTEC (Leman, France). Ring $^{13}C_6$-labeled L-tyrosine (99%) was purchased from ARC (Holland). Deuterations of the *p*-cresol ring were performed by acid-catalyzed exchange reactions essentially as previously described [42]. The labeling extent was estimated to be ≥90% by comparing the IR spectra of *p*-cresol-2,6-d_2, *p*-cresol-2,3,5,6-d_4 and *p*-cresol-3,5-d_2 in the 1800–600 cm^{-1} region with data in Ref. [42]. Phenol ^{18}O-labeling was done as described previously [43]. For synthesis of [1-^{18}O]*p*-cresol, diazonium sulphate was prepared by diazotation of *p*-toluidine followed by hydrolysis in boiling $H_2^{18}O$. The isotopic enrichment was 68% as determined by mass spectrometry.

2.2. FTIR spectroscopy

For the FTIR experiments, the molecules were dissolved in a borate buffer at the indicated pH and concentrations. Stable radicals were obtained by UV-irradiation (during 2 s) at 10 K [28]. The FTIR difference spectra were obtained by taking 128 scans before and after UV-irradiation. Spectra were recorded with 4 cm^{-1} resolution on a Bruker IFS 88 spectrometer equipped with a He-cooled cryostat.

3. Results

Fig. 1 shows the FTIR difference spectra obtained upon UV-irradiation of 10 mM solutions (at pH 12) of (a) tyrosinate (Tyr^{-}), (b) $^{2}H_4$-Tyr^{-}, c) $^{13}C_1$(4)-Tyr^{-} and d) $^{13}C_6$-Tyr^{-}, respectively. Spectrum a is very similar to that obtained previously with a 1 M solution of tyrosinate [28]. Spectrum a is also very similar to that obtained upon UV-irradiation of a 10 mM solution of *p*-cresol (Fig. 1e). In spectra of Fig. 1, negative bands correspond to the reduced species. In particular, the signals at 1606–1603, 1555–1554, 1501–1500, 1272–1267 and 1173 cm^{-1} in Fig. 1(a and e) are assigned to the ν_{8a}(CC), ν_{8b}(CC), ν_{19}(CC), $\nu_{7'a}$(CO), and 9a(CH) modes of tyrosinate or *p*-cresolate side chains, respectively, following the classification of Wilson [44] (see also Refs. [17,42,45,46]). The radical is characterized by one large positive IR band at 1513–1476 cm^{-1}. In our

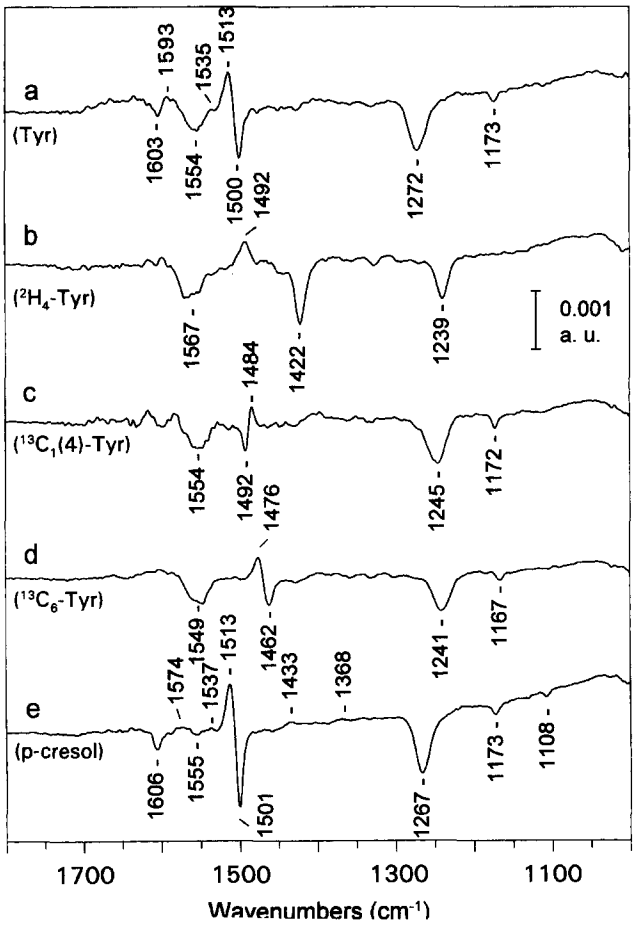

Fig. 1. UV-induced FTIR difference spectra: (a) Tyr$^{\cdot}$/Tyr^{-}; (b) $^2H_4$-Tyr$^{\cdot}$/$^2H_4$-Tyr^{-}; (c) $^{13}C_1$(4)-Tyr$^{\cdot}$/$^{13}C_1$(4)-Tyr^{-}; (d) $^{13}C_6$-Tyr$^{\cdot}$/$^{13}C_6$-Tyr^{-}; and (e) *p*-cresol$^{\cdot}$/*p*-cresol. Conditions: 10 mM solutions in 0.5 M borate buffer; pH 12; temperature, 10 K; resolution, 4 cm^{-1}. The spectra correspond to the average of data obtained with four to eight samples.

previous study, we observed a second feature at 1290 cm^{-1} in the UV-induced spectrum of phenol$^{\cdot}$ and *p*-ethylphenol$^{\cdot}$ [28]. We found, however, that the intensity of this signal is sensitive to the concentration of phenol or *p*-cresol in solution. From proton NMR experiments performed on phenol solutions at different pH and different phenol concentrations, we can now interpret this band as the $\nu_{7'a}$(CO) mode of a reduced phenol molecule interacting with phenol$^{\cdot}$. The absence of this band in Fig. 1e shows that such interactions are minimized with 10 mM solutions. Small positive bands at 1593–1585 and 1535 cm^{-1} for Tyr$^{\cdot}$ in Fig. 1a and at 1574, 1537, 1433, and 1368 cm^{-1} for *p*-cresol$^{\cdot}$ in Fig. 1e could also be radical modes. They are however at the detection limit. We also reported previously a combination mode for Tyr$^{\cdot}$ and phenol$^{\cdot}$ at ≈2110 cm^{-1} [28]. This band is still present in the spectra obtained with 10 mM concentration but with strongly reduced amplitude as compared to the 1513–1505 cm^{-1} band. It is not largely sensitive to deuteration of the *p*-cresol or phenol ring but it is downshifted by 26 cm^{-1} upon ^{18}O-labeling of phenol$^{\cdot}$ or *p*-cresol$^{\cdot}$ (not shown).

The frequency shift of the main IR mode of the radicals, observed between 1504 cm^{-1} and 1513 cm^{-1}, has been studied upon isotope-labeling. Downshifts by 37 cm^{-1} and 21 cm^{-1} are observed on the 1513 cm^{-1} signal of Tyr$^{\cdot}$ upon $^{13}C_6$- and $^2H_4$-labeling, respectively (Fig. 1d and b). These downshifts are comparable to those observed by RR spectroscopy [19] for the 1505 cm^{-1} band of phenol$^{\cdot}$. The large downshift induced by Tyr ^{13}C-labeling at the ring C4 carbon ($^{13}C_1$(4)-Tyr) on the 1513 cm^{-1} IR band (−29 cm^{-1}, Fig. 1c) is in agreement with its assignment to the ν(CO) mode of Tyr$^{\cdot}$.

Fig. 2 shows the effect of ^{18}O-labeling on the frequency of the ν(CO) mode in phenol$^{\cdot}$ (Fig. 2a) and *p*-cresol$^{\cdot}$ (Fig. 2b). Spectra with thin lines were obtained with ^{16}O-phenol and ^{16}O-*p*-cresol and those

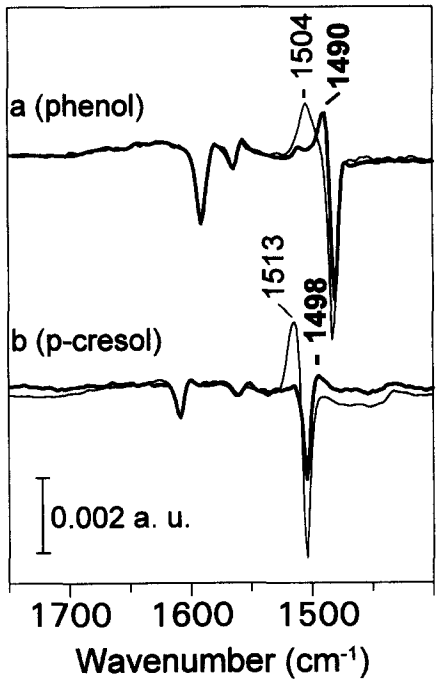

Fig. 2. UV-induced FTIR difference spectra: (a) ^{16}O-phenol$^{\cdot}$/^{16}O-phenol^{-}; thin line and ^{18}O-phenol$^{\cdot}$/^{18}O-phenol^{-}, bold line; (b) ^{16}O-*p*-cresol$^{\cdot}$/^{16}O-*p*-cresol^{-}, thin line and ^{18}O-*p*-cresol$^{\cdot}$/^{18}O-*p*-cresol^{-}, bold line. The contributions due to residual unlabeled phenol and *p*-cresol have been subtracted. Same instrumental conditions as in Fig. 1.

Table 1
Influence of isotope-labeling on the ν(CO) mode of phenolate and phenoxyl derivatives

	Unlabeled	^{18}O	2,6-$^2H_2$	3,5-$^2H_2$	$^2H_4$	$^2H_5$	$^{13}C_6$	$^{13}C_1$(4)
Phenolate	1273	1255 (−18)				1202 (−71)		
Cresolate	1268	1251 (−17)	1262 (−6)	1246 (−22)	1238 (−30)			
Tyrosinate	1272				1239 (−33)		1241 (−31)	1245 (−27)
	Unlabeled	^{18}O *(^{17}O)*	2,6-$^2H_2$	3,5-$^2H_2$	$^2H_4$	$^2H_5$	$^{13}C_6$	$^{13}C_1$(4)
Phenol$^{\cdot}$	1504	1490 (−14)				1486 (−18)		
	1505	*1492 (−13)*				*1490 (−15)*	*1469 (−36)*	
p-Cresol$^{\cdot}$	1513	1498 (−15)	1498 (−15)	1503 (−10)	1493 (−20)			
Tyr$^{\cdot}$	1513		1498 (−15)		1492 (−21)		1476 (−37)	1484 (−29)

Values in italic are taken from Ref. [19] and correspond to frequency detected by resonance Raman.

with bold lines were obtained with ^{18}O-phenol and ^{18}O-*p*-cresol, respectively. The positive bands at 1504 and 1513 cm^{-1} are downshifted by −14 and −15 cm^{-1}, respectively. Spectra in Fig. 1 also show the effect of isotope-labeling on the $\nu_{7'a}$(CO) mode of Tyr$^-$, phenolate and *p*-cresolate. The results are summarized in Table 1. Table 1 also reports the effect of (2,6)-$^2H_2$, $^2H_4$ and (3,5)-$^2H_2$-labeling of the *p*-cresol ring on the ν(CO) IR mode of *p*-cresol$^{\cdot}$ (spectra not shown).

4. Discussion

The isotope-induced frequency shifts on the Tyr$^{\cdot}$ model compounds reported in this study are close to those detected by RR spectroscopy (see Table 1). The effect of ^{18}O-labeling on both phenol$^{\cdot}$ and *p*-cresol$^{\cdot}$ (Fig. 2) demonstrates that the IR signals which are observed at 1504 or 1513 cm^{-1}, respectively, correspond to the ν(CO) mode of the radicals. The amplitude of the shift observed upon ^{18}O-labeling for phenol$^{\cdot}$ closely corresponds to that reported by RR upon ^{17}O-labeling of phenoxyl radical (Table 1) [19].

The shifts observed experimentally upon isotope-labeling can be used to test the validity of theoretical calculations on the radical structures [19]. In this respect, the 37 cm^{-1} downshift observed for the Tyr$^{\cdot}$ ν(CO) IR mode upon $^{13}C_6$-Tyr-labeling is in agreement with the predictions of density functional quantum chemical calculations on Tyr$^{\cdot}$ [38].

An important observation which follows from the FTIR in vitro study [23,28] is that one intense band (the ν(CO) mode at ≈1513 cm^{-1}) characterizes the IR spectrum of Tyr$^{\cdot}$. This IR mode, sensitive to ring substitution and isotope-labeling, can be identified in the complex FTIR difference spectra obtained in situ [23–25]. The ν(CO) mode was identified at 1503 cm^{-1} for the stable Tyr$_D^{\cdot}$ of PSII, using PSII with specifically labeled tyrosines [24,25]. In PSII obtained from a mutant in which Tyr$_D^{\cdot}$ is not hydrogen bonded, the ν(CO) mode of Tyr$_D^{\cdot}$ has been identified at 1498 cm^{-1} [24]. This frequency is similar to that observed in ribonucleotide reductase [11] for which the tyrosyl radical is not hydrogen bonded ([2] and Refs. therein). These data suggest that the frequency of the ν(CO) mode of Tyr$^{\cdot}$ could be a probe of hydrogen bond formation between Tyr$^{\cdot}$ and its environment in proteins.

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