Resonance Raman Studies of Catecholate and Phenolate Complexes of Recombinant Human Tyrosine Hydroxylase†

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ABSTRACT: Human tyrosine hydroxylase isoform 1 (hTH1) was expressed in *Escherichia coli*, purified as the apoenzyme, and reconstituted with iron. The resonance Raman spectra of hTH1 complexed with dopamine, noradrenaline, tyramine, and catechol have been studied and compared to those obtained for TH isolated from bovine adrenal glands or rat phaeochromocytoma tissue. A TH−phenolate complex is reported for the first time. Using dopamine selectively ^{18}O-labeled in the 3-position or both 3- and 4-hydroxy positions, we have been able to assign unambiguously the origin of the low-frequency vibration bands: the band at 631 cm^{-1} involves the oxygen in the 4-position; the band at 592 cm^{-1} involves the oxygen in the 3-position, and the band around 528 cm^{-1} is shifted by both, suggesting a chelated mode vibration. A small shift of the 1275 cm^{-1} band and no shift of the 1320 cm^{-1} band were observed, showing that those two bands involve essentially ring vibrations of the catecholate moiety, rather than the C−O stretching vibration as previously suggested. The spectrum of the catechol-d_6−hTH1 complex confirms this assignment. The resonance Raman spectra of the ^{54}Fe, ^{56}Fe, or ^{57}Fe isotope-containing enzymes complexed with dopamine are virtually identical, showing that the component of the iron in the ≈600 cm^{-1} vibrations is too small to be observed. These results provide a better understanding of the Raman properties of iron−catecholate complexes in this enzyme, as well as in other metalloproteins and model compounds.

Tyrosine hydroxylase (EC 1.14.16.2) catalyzes the rate-limiting step in the biosynthesis of catecholamines both in brain and in adrenal medulla (Levitt *et al.*, 1965). This enzyme contains non-heme iron and requires tetrahydrobiopterin as cosubstrate to hydroxylate L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA)[1] (Fukami *et al.*, 1990). A mechanism for the reaction requiring oxygen activation has been proposed (Dix *et al.*, 1987). Human tyrosine hydroxylase exists as four different isoforms (hTH1−hTH4) generated by alternative splicing events (Grima *et al.*, 1987; Nagatsu & Ichinose, 1991; O'Malley *et al.*, 1987). All isoforms have recently been expressed in *Escherichia coli* (Haavik *et al.*, 1991; Sutherland *et al.*, 1993). The recombinant enzymes are tetramers, composed of four identical subunits of predicted molecular masses (from the cDNA sequences) of 55 553−58 521 Da. The purified apoenzymes are rapidly activated (up to 40-fold) by the incorporation of 1 equiv of Fe^{2+} per subunit (Haavik *et al.*, 1991). The iron ligands are unknown, but spectroscopic studies [Mössbauer (Haavik *et al.*, 1993) and EXAFS (manuscript in preparation)] indicate that there is no tyrosine or sulfur coordination. Sequence comparisons among the different aromatic amino acid hydroxylases show that six histidines are conserved in the sequences (Grima *et al.*, 1987) of which some have been proposed as ligands. Together with the spectroscopic data, this suggests an environment of histidines and carboxylate(s) for the iron (Andersson *et al.*, 1988, 1992). The protein isolated from mammalian tissues has a blue−green color which has been shown to be due to an iron−catecholamine complex (Andersson *et al.*, 1988). Catecholamines act as feedback inhibitors of the catecholamine biosynthesis (Udenfriend *et al.*, 1965; Dairman & Udenfriend, 1971) by coordination to the TH active-site iron center (Andersson *et al.*, 1988, 1989). The biological significance of the catecholamine binding to TH has not been settled, and it is not yet clear whether such complexes are present *in vivo*, or are artifacts generated during enzyme purification, due to leakage of catecholamines from secretory granules. However, the enzyme as isolated from mammalian tissues contains nearly stoichiometric amounts of tightly bound catecholamines (Andersson *et al.*, 1988, 1992). When iron-reconstituted recombinant human TH is exposed to physiologically relevant concentrations of dopamine, noradrenaline, or

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[1] Abbreviations: hTH1, -2, -3, -4, human tyrosine hydroxylase isoforms 1, 2, 3, and 4, respectively; PAH, phenylalanine hydroxylase; 3,4-PCD, protocatechuate 3,4-dioxygenase; 1,2-CTD, catechol 1,2-dioxygenase; RNR R2, R2 protein of ribonucleotide reductase; LMCT, ligand-to-metal charge transfer; DOPA, 3,4-dihydroxyphenylalanine; PDA, *N*-(2-pyridylmethyl)iminodiacetate; salen, *N,N'*-ethylenebis(salicylidenimine) dianion.

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adrenaline, in the presence of oxygen, such complexes are also rapidly formed (Haavik *et al.*, 1991), in a reaction where the rate-limiting step appears to be the autoxidation of enzyme-bound Fe(II) to Fe(III). The binding of catecholamines to the enzyme influences many of the properties of the enzyme, including its catalytic activity, the affinity for iron, and the enzyme secondary structure as determined by FT-IR spectroscopy, rate of denaturation, substrate binding properties, and rate of proteolysis (Haavik *et al.*, 1991; Almås *et al.*, 1992; A. Martinez, personal communication). The inhibition of TH by catecholamines, which is competitive with respect to the tetrahydropterin cofactor, is partially reversed by phosphorylation of Ser-40. It has been suggested that this is the major mechanism for short-term regulation of TH *in vivo* (Haavik *et al.*, 1990).

Catechols can be very useful colorimetric probes for non-heme iron proteins and have been successfully used to study several non-heme iron proteins such as lipoxygenases (Cox *et al.*, 1988; Nelson, 1988), catechol dioxygenases (Que, 1983; Tyson, 1975), and ribonucleotide reductase (Ling *et al.*, 1994) (F208Y mutant of RNR R2 containing a catecholate motif chelated to the iron). In most cases, the catechol has been proposed to be chelated to the iron based on UV−visible and resonance Raman spectra and crystallographic data obtained on model compounds (Cox *et al.*, 1988; Heistand *et al.*, 1982). The blue−green catecholamine−TH complexes of the bovine (Andersson *et al.*, 1988) and rat (Andersson *et al.*, 1992) isolated enzymes have previously been studied by resonance Raman spectroscopy. The spectra exhibit characteristic features of catecholate−iron complexes with three bands around 600 cm^{-1} attributed to Fe−O stretching vibrations (Cox *et al.*, 1988), and two bands at 1272 and 1320 cm^{-1} assigned to C−O stretching vibrations (Cox *et al.*, 1988). However, a detailed isotopic labeling study has not been carried out to assign these resonance Raman vibrations. In this paper, we report resonance Raman studies of the recombinant human tyrosine hydroxylase isoform 1 (hTH1) complexed to dopamine, noradrenaline, catechol, and tyramine. We also describe an isotopic labeling study of the dopamine−hTH1 complex and the assignment of the vibration bands using enzymatically synthesized dopamine with ^{18}O either at position 3 or at positions 3 and 4 and enzyme containing ^{54}Fe, ^{56}Fe, or ^{57}Fe. These data allow a more precise assignment of the vibrational features arising from a catecholate complex and should be useful in interpreting the ligation modes of catechols bound to other non-heme iron metalloproteins.

MATERIALS AND METHODS

Human tyrosine hydroxylase isoform 1 (hTH1) was expressed in *E. coli* and purified to homogeneity as previously described (Haavik *et al.*, 1991). The purified preparations of hTH1 apoenzyme used in the present study contained 0.02 ± 0.01 atom of iron/subunit, as determined by atomic absorption spectroscopy. The concentration of purified hTH1 was determined by the absorbance at 280 nm ($\epsilon^{1\%} = 10.4$ cm^{-1}) at neutral pH (Haavik *et al.*, 1988). Tyrosine hydroxylase activity was assayed at 30 °C or 37 °C with L-[^{3}H]tyrosine and 6-methyltetrahydrobiopterin as the substrates as previously described (Haavik *et al.*, 1991). The phosphorylated hTH1 was prepared as previously described (Almås *et al.*, 1992). For the inhibition studies of hTH1 by tyramine and octopamine, the enzyme (3 μg/mL) was incubated with 100 μM iron(II) ammonium sulfate in 100 mM HEPES, pH 7.0, and either no inhibitor or 10 or 20 mM inhibitor. The initial rates were determined from the amount of tritiated water formed during a 6 min incubation time at 30 °C with either 25 μM [3,5-^{3}H]tyrosine and 20−125 μM 6(*R*)-tetrahydrobiopterin or 10−40 μM [3,5-^{3}H]-tyrosine and 500 mM 6(*R*)-tetrahydrobiopterin.

Preparation of Labeled Compounds. [3-^{18}O,4-^{16}O]Dopamine was prepared by incubating L-tyrosine (Sigma) with $^{18}O_2$ (96% ^{18}O; Cambridge Isotope Laboratories, Woburn, MA), 6-methyltetrahydropterin (Schircks Laboratories, Jona, Switzerland), dithiothreitol (Sigma), hTH1, catalase (Sigma), and tyrosine decarboxylase (Sigma) at pH 7.0 and isolating the product by HPLC, using a Partisil M9 (60 × 0.9 cm) strong cation-exchange column. [3-^{18}O,4-^{18}O]Dopamine was prepared similarly, but with L-phenylalanine (Sigma) as the amino acid substrate. [3-^{18}O, 4-^{18}O]Noradrenaline was prepared by incubating [3-^{18}O,4-^{18}O]dopamine with bovine adrenal dopamine β-hydroxylase (Sigma), ascorbate, catalase, dithiothreitol, and $^{16}O_2$ at pH 6.0 and isolating the product as described above. The identity and isotopic purity of the ^{18}O-labeled compounds were verified by HPLC−mass spectrometry analysis. Catechol was sublimed prior to use, and all other materials were reagent grade and used without further purification. Deuterated catechol (catechol-d_6) was obtained via base exchange (0.5 equiv of potassium *tert*-butoxide) at 140 °C for 20 h in a sealed tube. The reaction was monitored by ^{1}H-NMR spectroscopy and terminated when the spectrum showed >90% deuteration of the ring protons.

Preparation of Iron Isotopes. The $^{54}Fe^{II}Cl_2$ was prepared from $^{54}Fe_2O_3$ (95% ^{54}Fe) by heating the oxide with 50% HCl at 120 °C for 14 h, followed by drying under nitrogen, lyophilizing, dissolving in water, and reducing with 1.0−1.36 equiv of ascorbate. The $^{57}FeCl_2$ used was prepared from metallic ^{57}Fe (95% ^{57}Fe).

Preparation of the UV−Visible and Resonance Raman Samples. The apoenzyme (1 mM in subunits) was first reconstituted with stoichiometric amounts of Fe(II). Then the substituted phenols were added from concentrated buffered stock solution, and the complexation was followed by UV−visible spectroscopy. The Fe(III)−catecholate complexes formed spontaneously under aerobic conditions, as indicated by the appearance of the characteristic blue−green color, while the red-colored phenolate complexes were formed after the addition of hydrogen peroxide (added to about 2.5 mM from a 100 mM stock solution).

Resonance Raman Conditions. Resonance Raman spectra were obtained using a Spex Model 1403 spectrometer interfaced with a microcomputer for data collection and processing. Laser excitation was provided by a Spectra Physics Model 171 argon ion and 375B dye (Rhodamine 6G) lasers. The Raman scattering was collected at 90° with a slit width of 4 cm^{-1}. The samples were placed in a quartz spinning cell which was cooled to ca. 5 °C by blowing cold nitrogen gas on the cell. A typical sample consisted of 120 μL of an hTH1 solution (1 mM in subunits, 60 mg/mL) with 0.03−0.1 M sodium sulfate added as an internal standard. All data were referenced to the sulfate signal at 983 cm^{-1}. Samples were typically irradiated with ca. 50 mW of laser power. Excitation profiles were constructed by comparing peak heights relative to the sulfate standard.

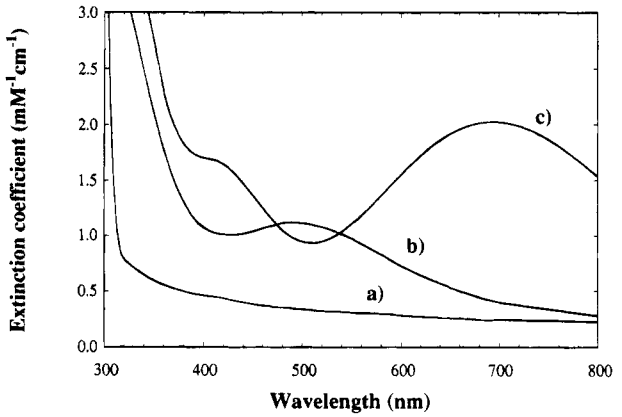

FIGURE 1: Visible absorption spectra of (a) the hTH1 apoprotein at 1 mM in the presence of 10 mM tyramine, (b) the hTH1–tyramine complex obtained from 1 mM hTH1 solution containing 10 mM tyramine, 1 mM iron(II) ammonium sulfate salt, and 2.5 mM H_2O_2, and (c) the hTH1–catechol complex obtained by stoichiometric addition of catechol to the 1 mM hTH1 solution. These spectra were recorded at room temperature in 50 mM phosphate buffer at pH 7.3.

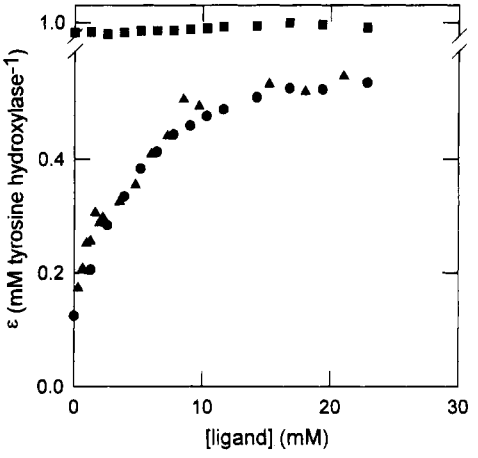

FIGURE 2: Titration of hTH1 with octopamine (▲) or tyramine (●) monitored at 520 nm using apoenzyme (1.25 mM in subunits) incubated with 1.25 mM iron(II) ammonium sulfate and 1.25 mM hydrogen peroxide in 20 mM HEPES buffer, pH 7.2. The absorbance at 416 nm as a function of added tyramine was also measured (■).

RESULTS

Complexes of hTH1 with Phenolates. Recombinant TH cloned and overexpressed in *E. coli* is purified as a colorless apoprotein (Haavik *et al.*, 1992) (Figure 1a). No new visible chromophore is observed after the addition of Fe(II) to the protein (Haavik *et al.*, 1992), but a faint yellow color appears after treatment with H_2O_2 to oxidize the Fe(II) to Fe(III) (data not shown). The Fe(III) enzyme becomes more strongly colored after binding phenols (red) or catechols (blue–green). Spectra for the hTH1 complexed with tyramine and catechol are shown in Figure 1b,c, respectively.

The red chromophore can be obtained with phenol, as well as aminoalkylphenols such as *p*-tyramine and *p*-octopamine. The latter compounds are trace amines present in the nervous system of many organisms (Nguyen & Juorio, 1989; Andrew *et al.*, 1993) and are substrate analogs of TH. Steady-state kinetic studies show that both compounds inhibit TH competitively with respect to the pterin cofactor and noncompetitively with respect to L-tyrosine (K_i = 3 mM for both compounds, Figure 2). Furthermore, the binding of the phenols and catechols to TH was shown to be mutually

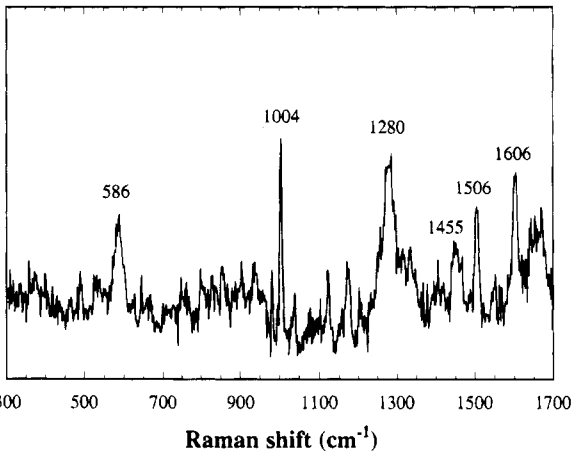

FIGURE 3: Resonance Raman spectrum of the tyramine–hTH1 complex obtained on a ≈1 mM sample (120 μL) in a spinning cell at 5 °C (90° scattering geometry) using a 514.5 nm excitation wavelength, a scan rate of 2 cm^{-1}/s, a 4 cm^{-1} slit, and accumulation of 16 scans. The contributions from the buffer (0.2 M MES, pH 6.5) and free tyramine (10 mM) have been subtracted from the spectrum.

exclusive (data not shown), indicating overlapping binding sites.

The binding of these phenol inhibitors to Fe^{III}hTH1 can be monitored spectrophotometrically by the appearance of an absorption band around 500 nm with an extinction coefficient of about 1.1 mM^{-1} cm^{-1}. Such a feature is similar to those found for a number of iron–tyrosinate proteins, and the extinction coefficient suggests a 1:1 stoichiometry for the complex (Pyrz *et al.*, 1985). Figure 2 shows the concentration dependence of chromophore formation observed at 520 nm on tyramine and octopamine, while the reference wavelength 416 nm shows no increase at all. The K_d values for both tyramine and octopamine are estimated to be 2.5 ± 0.5 mM. Laser excitation into this visible chromophore reveals resonance-enhanced Raman vibrations at 586, 1280, 1506, and 1606 cm^{-1}, which are characteristic of iron(III) complexes with para-substituted phenolates (Figure 3, Table 1) (Que, 1983, 1988). The 586 cm^{-1} band is associated with the Fe–OR stretching mode, while the bands at 1280, 1505, and 1605 cm^{-1} correspond to phenolate ring deformations. These data demonstrate that tyramine coordinates to the Fe(III) center in tyrosine hydroxylase.

Unlike tyramine and octopamine, tyrosine does not appear to elicit these spectral changes with hTH1; no LMCT band develops at tyrosine concentrations up to 3 mM, and the presence of 1 mM tyrosine does not interfere with the binding of catechols to the enzyme. However, tyrosine has been reported to inhibit rat TH (Fitzpatrick, 1991) and hTH1 (Martinez *et al.*, 1993) at concentrations above 50 μM. It has been proposed that this inhibition could be due to the binding of the substrate prior to the binding of the pterin, forming a dead-end enzyme–tyrosine complex (Fitzpatrick, 1991). The lack of a visible chromophore upon addition of millimolar concentrations of tyrosine suggests that the tyrosine in the dead-end complex does not bind to the enzyme-bound iron, but binds to the enzyme in a different mode.

Complexes of hTH1 with Catechols. Like tyramine and octopamine, catecholamines inhibit TH activity ($K_i \approx 0.5$–1 μM) competitively with respect to the pterin cofactor (Le Bourdellès *et al.*, 1991) and noncompetitively with respect

Table 1: Resonance Raman Vibrations of Iron–Catecholate and Iron–Phenolate Chromophores in Proteins

complexes	excitation wavelength (nm)	Raman vibrations (cm^{-1})										
TH–dopamine[a,b]	606	528	592	631		1275	1320	1425	1475			
phosphorylated TH–dopamine[b]	606	531	584	638		1270	1320	1424	1478			
TH–noradrenaline[b]	606	530	624	636	1171	1271	1328 (br)	1428	1476			
TH–catechol[b]	606	528		619	1150	1257	1314		1466			1566
rat tumor TH[c]	605	527	589	633		1272	1321	1426	1475			
bovine TH[d]	605	527	604	635	1130	1271	1320	1426	1476			
PAH–catechol[e]	620.5	531		621	1151	1257	1313		1470			1568
TH–tyramine[a]	514.5		589		1175	1281				1507		1605
1,2-CTD	514.5[f]				1174	1255–1274				1506		1605
	647[g]				1173	1293				1505		1605

[a] Sample prepared with ^{56}Fe. [b] Sample prepared with ^{54}Fe. [c] Andersson *et al.* (1992). [d] Andersson *et al.* (1988). [e] Cox *et al.* (1988). [f] Michaud-Soret, I., unpublished results on purified 1,2-CTD isozymes. [g] Que *et al.* (1980).

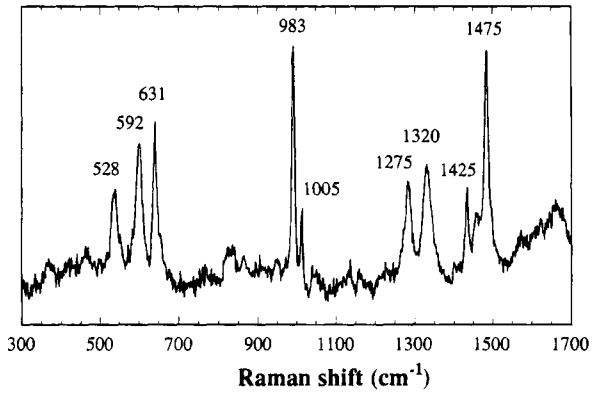

FIGURE 4: Resonance Raman spectrum of the dopamine–hTH1 complex obtained on a ≈1 mM sample (120 μL) in a spinning cell at 5 °C (90° scattering geometry) using a 606 nm excitation wavelength, a scan rate of 2 cm^{-1}/s, a 4 cm^{-1} slit, and accumulation of 40 scans.

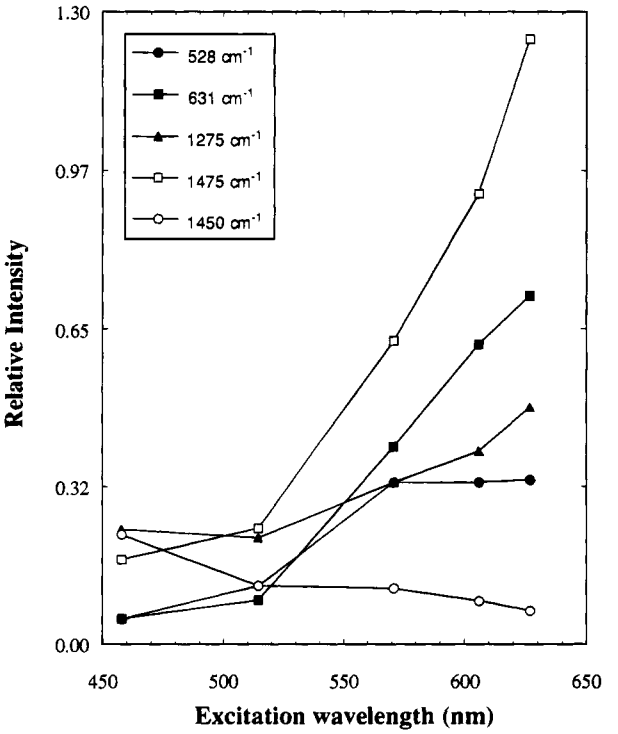

FIGURE 5: Excitation profiles for the dopamine–hTH1 complex sample relative to the sulfate standard. The spectra were obtained on a ≈1 mM sample (120 μL) in a spinning cell at 5 °C (90° scattering geometry) using 457.9, 514.5, 571, 606, and 627 nm excitation wavelengths (≈50 mW), a scan rate of 2 cm^{-1}/s, a 4 cm^{-1} slit, and accumulation of 7–40 scans.

to the substrate (Almås *et al.*, 1992). The K_d for dopamine binding to the iron-reconstituted hTH1 is in the 2–3 μM range (Almås *et al.*, 1992).

The visible spectrum of Fe^{III}hTH1 complexes with catechol is almost identical to that previously described for the complex with dopamine (Haavik *et al.*, 1991). Catechol, dopamine, and noradrenaline all form 1:1 complexes with hTH1 with absorption maxima at 415–420 and 695–700 nm (Figure 1c) due to catecholate LMCT bands. The spectra of the enzyme–catecholate complexes are also very similar to those of the catechol complex of the rat liver phenylalanine hydroxylase and tyrosine hydroxylases as isolated from rat phaeochromocytoma and bovine adrenal glands (Haavik *et al.*, 1988; Andersson *et al.*, 1992).

The resonance Raman spectrum of the hTH1-dopamine complex (Figure 4) has the characteristic signature of an iron–catecholate species (Cox *et al.*, 1988; Pyrz *et al.*, 1985) with bands at 528, 592, 631, 1275, 1320, 1425, and 1475 cm^{-1}. The spectrum is almost identical to the one obtained from the isolated PC12 TH (Andersson *et al.*, 1992). These spectral features are also similar to the Fe–DOPA complex recently described in the mutant F208Y ribonucleotide reductase (RNR) R2 protein (Ling *et al.*, 1994). The Raman spectrum of the hTH1 complex with noradrenaline (Table 1) shows subtle differences from that of the dopamine complex in the region around 600 cm^{-1}. The same differences are observed in the spectra of Fe(PDA) complexed with dopamine and noradrenaline (data not shown), suggesting that these differences are derived from the presence of the hydroxyl substituent on the side chain. The excitation profile of the hTH1–dopamine complex (Figure 5) confirms that all the bands except those at 1005 and 1450 cm^{-1} are related to the catecholate–iron LMCT band at 700 nm (Cox *et al.*, 1988). The 1005 cm^{-1} band is not Raman-enhanced and is assigned to a phenylalanine ring mode from the protein (Lord & Yu, 1970). Since the 1450 cm^{-1} band appears to increase in intensity with 457.9 nm excitation, it might be associated with the higher energy absorption band at 420 nm, as has been observed in model compounds (Cox *et al.*, 1988).

Isotopic Labeling Studies of hTH1–Catechol Complexes (^{18}O, ^{54}Fe, ^{57}Fe, and ^{2}H). In the resonance Raman studies on iron(III)–catecholate complexes thus far published, the assignments for most of the observed vibrations have been assumed to follow assignments made on the corresponding phenolate complexes. Thus, the bands in the 1200–1500 cm^{-1} region are associated with ring deformation modes, the ca. 1270 cm^{-1} band in particular being assigned to the catecholate C–O stretch. The bands around 600 cm^{-1}, on the other hand, are associated with metal–ligand vibrations, the 528 cm^{-1} band in particular being assigned to a chelate

Table 2: Effects of Isotopic Labeling on hTH1−Catecholate Modes

complexes	Raman vibrations (cm^{-1}) (excitation wavelength at 606 nm)								
[3-^{16}O,4-^{16}O]dopamine	528	592	631		1275	1320	1425	1475	
[3-^{18}O,4-^{16}O]dopamine	522	580	629		1271	1320	1425	1475	
[3-^{18}O,4-^{18}O]dopamine	509	578	619		1266 (br)	1320	1423	1473	
[3-^{16}O,4-^{16}O]noradrenaline	530	624	636	1170	1271	1328 (br)	1428	1476	
[3-^{18}O,4-^{18}O]noradrenaline	512	552−597	624	1168	1266	1317	1424	1474	
catechol	528	619		1150	1257	1314		1466	1566
catechol-d_6	522	609		1130	1200		1414		1536
DOPA208 R2[a]	512	592	619	1143	1263	1319	1414	1475	1569
[3-^{18}O]DOPA208 R2[a]	499	584	617	1143	1263	1319	1414	1475	1569

[a] Ling et al. (1994).

mode by analogy to a similar band observed in the spectrum of $[Fe(oxalate)_3]^{3-}$ (Nakamoto, 1978). No detailed isotopic labeling study has been carried out, however. The ease of obtaining ^{18}O-labeled catechols from TH-catalyzed reactions prompted us to extend our Raman studies to these complexes.

Resonance Raman spectra of TH complexed with ^{18}O-labeled catechols showed the same pattern of signals as the ^{16}O derivatives. Table 2 and Figure 6 compare the data for hTH1 complexed with dopamine with (a) 3-^{16}O,4-^{16}O, (b) 3-^{18}O,4-^{16}O, and (c) 3-^{18}O,4-^{18}O. In the high-energy region, only the band at 1275 cm^{-1} is affected upon oxygen labeling in the 3- and 3,4-positions ($\Delta\nu$ of −4 and −9 cm^{-1}, respectively); there is no observable shift for the bands at 1320 and 1475 cm^{-1}. Thus, only the 1275 cm^{-1} vibration includes a C−O stretching component, and its contribution is rather small. The 1320 and 1475 cm^{-1} features must involve deformations that do not have a C−O stretching component. The vibrations in the low-energy region, on the other hand, are significantly affected by the presence of ^{18}O. Our data (Figure 6) allow us to conclude unambiguously that: (1) all three bands have a large oxygen component, (2) the band at 631 cm^{-1} is associated with the oxygen at C-4, (3) the mode at 592 cm^{-1} involves the oxygen at C-3, (4) the band around 528 cm^{-1} involves both. Similar shifts are observed for the three low-energy features in the doubly ^{18}O-labeled noradrenaline complex (Table 2) and the Fe^{III}-DOPA complex in RNR R2(F208Y) where the 3-OH group was labeled with ^{18}O (Ling *et al.*, 1994).

To complement the ^{18}O isotope data, we examined a complex with ring-deuterated catechol. The Raman spectrum of the hTH1−catechol complex is similar to that of the hTH1−dopamine spectrum. The principal difference appears in the low-energy region (see Table 1), where only two bands are observed at 528 and 619 cm^{-1} with a 1:2 intensity ratio, compared to the 1:1:1 intensity ratio observed in the complexes with 4-substituted catechols. The two bands near 600 cm^{-1} merged into one feature due to the 2-fold symmetry of catechol. Ring deuteration of the catechol results in the disappearance of the 1314 cm^{-1} band and significant downshift of the other bands (Table 2) as previously reported for resonance Raman spectra of iron−catecholate model compounds (Pyrz *et al.*, 1985) as well as in Raman spectra of catechol-d_6 (Greaves & Griffith, 1991). Thus, all the observed vibrations have substantial ring deformation components.

In contrast, no differences could be observed in the entire spectral region of interest for samples with incorporated ^{54}Fe and ^{57}Fe labels; furthermore, no broadening of spectral features in a sample with a 50:50 ^{54}Fe:^{57}Fe ratio was detected. These data suggest that the contribution of the iron to these vibrations is too small to be detected under our instrumental conditions. This result, however, is not surprising, since the ^{54}Fe/^{57}Fe isotope shift expected for a pure Fe-O stretch at 600 cm^{-1} is no more than 4 cm^{-1} and the ^{16}O/^{18}O and ^{1}H/^{2}H isotope shifts indicate that these vibrations have significant aromatic ring character.

Resonance Raman Spectra of Dopamine Complexed with the Phosphorylated Form of the hTH1. hTH1 has been shown to be activated on phosphorylation by c-AMP-dependent protein kinase (on Ser-40) or calmodulin-dependent protein kinase II (on Ser-19 and Ser-40) (Le Bourdellès *et al.*, 1991). This activation is due to an increase in the affinity of the cofactor tetrahydrobiopterin (BH_4), which is proposed to bind close to the iron center during the catalytic cycle (Martínez *et al.*, 1993b). Extrinsic fluorescence and FT-IR spectroscopy have demonstrated changes in the secondary structure on phosphorylation (Roskoski *et al.*, 1993; A. Martinez, personal communication). The rate of dissociation and the K_d values of catecholamines are also increased on phosphorylation (Almås *et al.*, 1992; Haavik *et al.*, 1990). As the hTH1 enzyme is isolated in a nonphosphorylated form, it is interesting to compare the resonance Raman spectra of the dopamine complexes of phosphorylated and nonphosphorylated enzyme. Phosphorylation engenders changes in the 600 cm^{-1} region (Table 1). The persistence of the ca. 530 cm^{-1} feature indicates that the catechol remains chelated to the metal center. It is intriguing to note the downshift of the vibration associated with the 3-OH moiety and the corresponding upshift of the mode associated with the 4-OH group. These shifts suggest a change in the way dopamine interacts with the active-site metal center, perhaps to a more unsymmetric bidentate mode favoring a stronger interaction between the Fe and the 3-O group.

DISCUSSION

Here we have shown that phenols and catechols have access to the iron site of oxidized hTH1 and form complexes with the enzyme-bound Fe(III) to give rise to LMCT bands in the visible region. Laser excitation into these LMCT bands afforded characteristic resonance-enhanced Raman vibrations typical of phenolates and catecholates. The availability of isotopically labeled catecholamines from the reaction of hTH1 with suitable substrates allowed us to explore in some detail the resonance-enhanced catecholate vibrations attributed to Fe−O and C−O modes.

Of the many bands observed in the resonance Raman spectra of the hTH1−catecholate complexes, we have found that the bands in the 600 cm^{-1} region provide the best insight

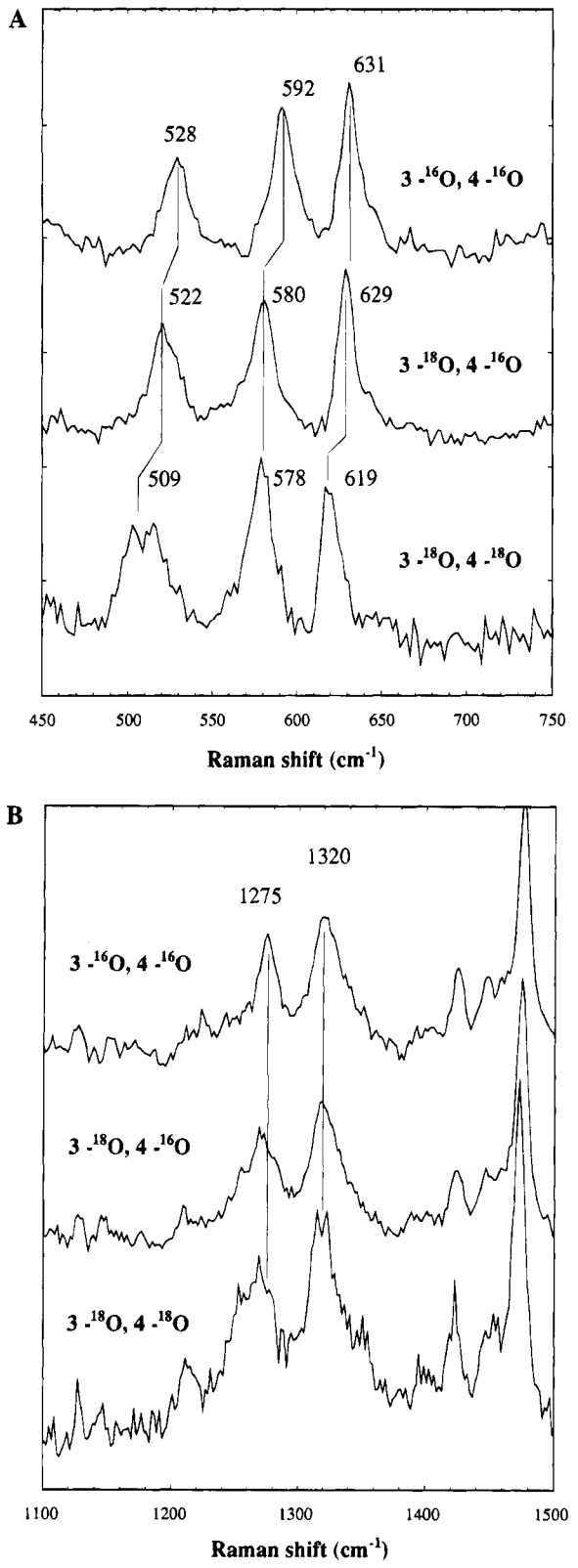

FIGURE 6: Raman spectra of ^{18}O-labeled dopamine−hTH1 complex. Stoichiometric amounts of ^{18}O-labeled dopamine were complexed to hTH1. The spectra were obtained on ≈1 mM samples (120 μL) in a spinning cell at 5 °C (90° scattering geometry) using a 606 nm excitation wavelength, a scan rate of 2 cm^{-1}/s, a 4 cm^{-1} slit, and accumulation of 60 scans.

into the coordination chemistry of the bound catecholate. This region has not previously been investigated in detail. Iron−tyrosinate proteins exhibit a band in this region that is associated with the ν(Fe−O) vibration. Examples include mutant hemoglobins where the proximal histidine has been replaced by tyrosine (HbM Iwate, 589 cm^{-1}, HbM Boston, 603 cm^{-1}; Nagai *et al.*, 1983) and purple acid phosphatases (575 cm^{-1}; Antanaitis *et al.*, 1982; Averill *et al.*, 1987). These are similar to the 567 cm^{-1} band corresponding to the ν-(Fe−O_2) stretch of oxyhemoglobin which has been unequivocally assigned by its shift to 540 cm^{-1} upon $^{18}O_2$ substitution (Brunner, 1974). A detailed Raman study on the model compound Fe(salen)−OC_6H_4-4-CH_3 using ^{18}O-labeled or ring-deuterated *p*-cresol has assigned the observed 570 cm^{-1} band to ν(Fe−OAr), *i.e.*, a combination of the Fe−O stretch and ν_{12} of a para-disubstituted benzene ring (Pyrz *et al.*, 1985). In the TH−tyramine complex, this vibration is found at 586 cm^{-1}.

With the availability of specifically ^{18}O-labeled dopamine, we can unequivocally assign the features in the 600 cm^{-1} region of the hTH1−catechol complexes to deformations associated with the Fe−enediolate unit. In contrast to the solitary band observed in the tyramine complex, two bands at 528 and 619 cm^{-1} are found in the TH−catechol complex; these bands have a 1:2 relative intensity ratio. On the other hand, three bands of equal intensity are seen in the TH complexes of catecholates with 4-substituents. Our ^{18}O-labeling studies show that the ca. 520 cm^{-1} feature shifts when either catecholate oxygen is labeled. This confirms the assignment of this vibration to a five-membered ring chelate mode made earlier on the basis of comparisons of the spectra of $[Fe(cat)_3]^{3-}$ (Salama *et al.*, 1978) and $[Fe(oxalate)_3]^{3-}$ (Nakamoto, 1978). A normal coordinate analysis of the oxalate complex has assigned this band as a mode intrinsic to the five-membered ring upon chelation of the oxalate to the iron (Nakamoto, 1970), and this assignment has been extended to the catecholate complexes (Salama *et al.*, 1978). Similarly, the presence of this band in the spectra of the TH complexes with dopamine, catechol, and noradrenaline allows us to conclude that they are chelated to the iron in the enzyme. Our ^{18}O-labeling studies also demonstrate unequivocally that the remaining two vibrations near 600 cm^{-1} correspond to modes that involve one catecholate oxygen or the other but not both. Our observations are corroborated by the results of Ling *et al.* (1994) on the Fe^{III}-DOPA complex in RNR R2(F208Y) with ^{18}O on the 3-OH of DOPA208. These results indicate that this region can be useful for deducing the catecholate coordination mode in other enzymes such as the catechol dioxygenases.

Our results call into question the nature of the 1260 and 1320 cm^{-1} vibrations which are commonly attributed to the catecholate ν(C−O) modes. The small shift observed for the 1260 cm^{-1} band and the lack of one for the 1320 cm^{-1} band upon ^{18}O incorporation into the catecholate oxygens argue against such an assignment. Indeed, the nearly 60 cm^{-1} shift observed when ring-deuterated catechol was used in the study of $[Fe(cat)_3]^{3-}$ strongly suggests that these bands are in fact ring vibrations of ortho-disubstituted benzenes, with at best a small contribution from the catecholate C−O stretches. For example, there are three ring vibrations (ν3, ν7a, and ν13) for o-xylene, catechol, or *o*-cresol which occur in the 1200−1300 cm^{-1} range, where ν7a is usually a strong polarized band and ν3 and ν13 are medium or weak intensity bands (Varsányi, 1974). These bands have been called the C−X bands of substituted benzenes in the literature (Varsányi & Szoke, 1969) and subsequently became mistakenly associated solely with the ν(C−O) (Wilson, 1974). Despite this misconception, these bands remain a characteristic spectroscopic signature for metal−catecholate coordination, being resonance-enhanced when the catecholate-to-iron(III)

charge-transfer chromophore is excited. The 1320 cm^{-1} band in particular is distinct from any feature observed for the iron−tyrosinate chromophore and thus useful for distinguishing phenolate from catecholate coordination.

The binding of both phenols and catechols to hTH1 to form these complexes with LMCT bands depends on the rate of oxidation of Fe(II) to Fe(III). While catechols can catalyze this autoxidation at neutral pH and under aerobic conditions, phenols cannot. In the latter case, the Fe(II) must first be oxidized by H_2O_2 in order for the LMCT complexes with phenols to form. We have previously shown that the binding of tyrosine to bovine TH causes subtle changes in the EPR spectra of the enzyme-bound Fe(III) (Andersson *et al.*, 1989). Thus, one could speculate that tyrosine, the normal amino acid substrate of TH, also binds to the iron center during the catalytic cycle. This hypothesis would be in accordance with the recently proposed mechanism for phenolate hydroxylation with model compounds (Kitajima *et al.*, 1993). However, this hypothesis does not explain the comparable rates of hydroxylation of phenylalanine and tyrosine by TH (Fukami *et al.*, 1990; Martínez *et al.*, 1993a). Moreover, steady-state kinetic analyses showed that the phenols tested (tyramine and octopamine) were noncompetitive inhibitors with respect to tyrosine and competitive inhibitors with respect to tetrahydrobiopterin, and no LMCT- band was observed at tyrosine concentrations up to 3 mM (concentration limited by the solubility of tyrosine). Recent ^{1}H-NMR data have also shown that the ring protons of phenylalanine are 6.6−6.8 Å from the metal center of Co(II)-substituted hTH1 (Martínez *et al.*, 1993a), while those of 6-methyltetrahydropterin are only 3.8−4.6 Å from the metal center, allowing direct coordination of the pterin to the metal center (Martínez *et al.*, 1993b). Thus, the inhibition observed at high concentrations of tyrosine is very likely not due to direct interaction with the iron center.

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