Evidence for Retention of Biological Activity of a Non-Heme Iron Enzyme Adsorbed on a Silver Colloid: A Surface-Enhanced Resonance Raman Scattering Study†

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ABSTRACT: The structure and catalytic properties of the enzyme (E) chlorocatechol dioxygenase (CCD) adsorbed on a citrate-reduced silver colloid are analyzed by surface-enhanced resonance Raman spectroscopy (SERRS). This is the first SERRS study of a non-heme metalloenzyme. It is demonstrated that the native conformation of CCD is retained in the adsorbed state by comparison of resonance Raman scattering (RRS) from CCD in solution with SERRS from CCD adsorbed on the silver colloid. Both spectra show clear evidence of vibrational bands typical of iron–tyrosinate proteins. Furthermore, it is demonstrated that adsorbed CCD retains 60–85% of its enzymatic activity in the reaction of catechol substrate (S) with O_2 to give the dioxygenated product (P) *cis,cis*-muconate. This is accomplished by enzyme assays of Ag-adsorbed CCD and comparison of the SERRS of Ag-adsorbed enzyme–substrate (ES) complex under anaerobic conditions with that of Ag-adsorbed ES in the presence of dioxygen. The SERRS difference spectrum, ES(aerobic) – ES(anaerobic), shows clear evidence for the appearance of the vibrational modes of adsorbed product. The analogous SERR difference spectroscopy experiment is also carried out for the enzyme–inhibitor (EI) complex of CCD with tetrachlorocatechol (TCC). Slow turnover of CCD–TCC is observed by SERRS on exposure to dioxygen which is consistent with the slow rate of turnover of TCC by CCD in solution.

Many useful principles of chemical catalysis have been elucidated by the spectroscopic study of intermediates in enzymatic reaction mechanisms; however, physical methods that are selective enough to distinguish between enzyme–substrate (ES)[1] complexes, enzyme-bound intermediates, and enzyme–product (EP) complexes typically suffer from a lack of sensitivity. The catechol dioxygenases, non-heme iron enzymes which catalyze the intradiol addition of oxygen directly to catechols causing fission of the aromatic ring, are a case in point. Despite the large body of work on these enzymes including the structural characterization of the protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa* (Ohlendorf et al., 1988), the nature of the intermediates in the catalytic mechanism of oxygen addition remain in question. In the studies performed to date on the intradiol catechol dioxygenases, the first intermediate (ES), is stable in the absence of O_2. This complex has been suggested to be either a monodendate or bidendate iron–catechol complex with three or four additional protein side chains serving as ligands. Stopped-flow methods using electronic absorption spectroscopy have provided insight into the reaction intermediates of these enzymes (Bull et al., 1981; Walsh & Ballou, 1983; Walsh et al., 1983). While electronic absorption spectroscopy can be indicative of tyrosine or catechol coordination to high spin Fe(III), it is not otherwise diagnostic of the chemical environment of the iron and, thus, is only of limited value in defining the nature of the intermediates.

Detailed information about the structure and dynamics of enzymatic reaction intermediates is potentially available from advanced vibrational spectroscopic methods such as resonance Raman spectroscopy (RRS) (Spiro, 1988; Spiro et al., 1990), ultraviolet RRS (UVRRS) (Spiro et al., 1990; Austin et al., 1993), and time-resolved RRS (TR^3S) (Petrich & Martin, 1989). RRS is both a very sensitive and selective spectroscopic probe. The intensity of RR scattering is well-known to be 10^3–10^4 times greater than that of nonresonant or normal Raman scattering (NRS) due to visible or ultraviolet laser excitation within an electronic absorption band of the target species. As a consequence, high signal-to-noise ratio (S/N) vibrational spectra can be readily obtained from dilute solutions (viz., 10^{-3}–10^{-6} M) of biological molecules. Whether RRS has millimolar or micromolar sensitivity for a particular target molecule depends on the value of its molar extinction coefficient at the laser excitation wavelength and the magnitude of its polarizability tensor elements.

For the chlorocatechol dioxygenase (CCD) systems being considered here, we anticipate that although the selectivity of RRS will be quite adequate for distinguishing the signals from CCD and several proposed intermediates, including semiquinone–Fe(II)–O_2, the sensitivity of RRS is unlikely to be adequate. This is a result of the rapid kinetics involved in the turnover of substrates by CCD, such that any transient intermediates are likely to be present only at submicromolar

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[1] Abbreviations: E, enzyme; CCD, chlorocatechol dioxygenase; SERRS, surface-enhanced resonance Raman scattering or spectroscopy; RRS, resonance Raman scattering or spectroscopy; S, substrate; P, product; ES, enzyme substrate; EI, enzyme inhibitor; TCC, tetrachlorocatechol; EP, enzyme product; UVRRS, ultraviolet resonance Raman spectroscopy; TR^3S, time-resolved resonance Raman spectroscopy; NRS, normal Raman scattering or spectroscopy; S/N, signal to noise ratio; Mb, myoglobin; Hb, hemoglobin; LMCT, ligand-to-metal charge transfer.

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concentrations. In addition, there are likely to be the usual interference problems caused by low level intrinsic fluorescence from a biological chromophore. This set of experimental circumstances is not unique to the CCD system. RRS studies of many biological molecules cannot be undertaken because it is either impossible or impractical to prepare 10^{-3}–10^{-6} M solutions as a result of scarcity of material or low solubility.

As a consequence, we are beginning the development of surface-enhanced resonance Raman scattering (SERRS) for the study of CCD and its complexes with substrates and products. SERRS has sufficient sensitivity and selectivity to produce high S/N spectra from a wide variety of biological molecules at micromolar to nanomolar concentration levels (Cotton, 1985, 1988; Koglin & Sèquaris, 1986; Nabiev et al., 1988, 1993; Cotton et al., 1991a; Fabian & Anzenbacher, 1993). However, the central question governing the biological relevance of SERRS has not yet been fully answered. This question is, Do the spectra observed in a SERRS experiment result from molecules that retain their native conformation and biological activity or do they result from denatured and bioinactive molecules?

There is clear evidence in SERRS studies of some proteins that denaturation does in fact occur upon interaction of the biomolecule with the SERS-active surface. Myoglobin (Mb) is very sensitive to the nature of the Ag surface (Cotton et al., 1980, 1991b). In hemoglobin (Hb), the heme can be dissociated from the protein accompanied by formation of μ-oxo dimers when borohydride reduced Ag colloids are used as the enhancing surface (Smulevich & Spiro, 1985). The flavoproteins suffer loss of flavin upon contact with SERS-active Ag surfaces in all cases reported to date (Copeland et al., 1984; Cotton & Holt, 1985; Lee, N.-S., et al., 1986, 1987; Holt & Cotton, 1987, 1989; Brabec & Niki, 1988).

There is also strong evidence for the retention of native conformation based on the comparison of SERRS vs RRS and redox potentials in adsorbed vs solution states (Hildebrandt & Stockburger, 1986, 1989; Kelly et al., 1987; Niki et al., 1987; Hildebrandt et al., 1988; Wolf et al., 1988; Cotton et al., 1989, 1991b; Hashimoto et al., 1989; Rospendowski et al., 1989, 1991). In addition, retention of biological activity has been indicated. Verma and co-workers (Verma et al., 1989) demonstrated that adsorbed cytochrome c_3 could be reduced by hydrogen in the presence of its physiological reduction partner, hydrogenase. De Groot and Hester (1988) obtained the SERR spectrum of native Hb when citrate-reduced Ag colloids were used as the enhancing surface and showed (1) reversible dioxygen and carbon monoxide binding and (2) a reversible R- to T-state transition.

Thus it appears that retention of native conformation and biological activity cannot be predicted *a priori* in SERRS. In this communication, we describe an advance in the application of SERRS to the study of the structure and function of adsorbed biological molecules. This is the first SERRS study in which the biological activity of a Ag-adsorbed enzyme is directly monitored by the vibrational spectroscopy of its complexes with substrates, inhibitors, and products. This dioxygenase enzyme has several features which can be contrasted with the more commonly studied heme proteins. First, CCD has a broad (390–700 nm) tyrosinate to Fe(III) ligand to metal charge transfer (LMCT) transition centered at λ_{max} = 430 nm with a molar extinction coefficient, ϵ_{430} = 3095 M^{-1} cm^{-1}. At the primary laser excitation wavelength, λ_{ex} = 514.5 nm, used in this study, CCD has $\epsilon_{514.5} \sim 2900$ M^{-1} cm^{-1}. Thus the LMCT transition of CCD is much less strongly absorbing than the transitions typically used for RRS and SERRS in the heme proteins. Taking Fe(II) cytochrome *c* as a case in point, its Soret (λ_{max} = 410 nm) and α,β transitions (λ_{max} = 520 and 550 nm) have molar extinction coefficients of ϵ_{410} = 106 100 M^{-1} cm^{-1}, ϵ_{520} = 15 900 M^{-1} cm^{-1}, ϵ_{550} = 27 700 M^{-1} cm^{-1}, and at λ_{ex} = 514.5 nm ϵ_{515} = 12 600 M^{-1} cm^{-1} (Margoliash et al., 1959). Thus the successful acquisition of spectra for CCD extends the range of applicability of SERRS to more weakly absorbing chromophores. Secondly, CCD, unlike the heme and flavoproteins, contains a covalently bound chromophore which cannot be removed from the protein without its destruction. Therefore, SERRS observation of the characteristic tyrosinate vibrational modes that are resonance-enhanced via the LMCT transition of the Fe(III)–tyrosinate center is an indicator for retention of native conformation.

MATERIALS AND METHODS

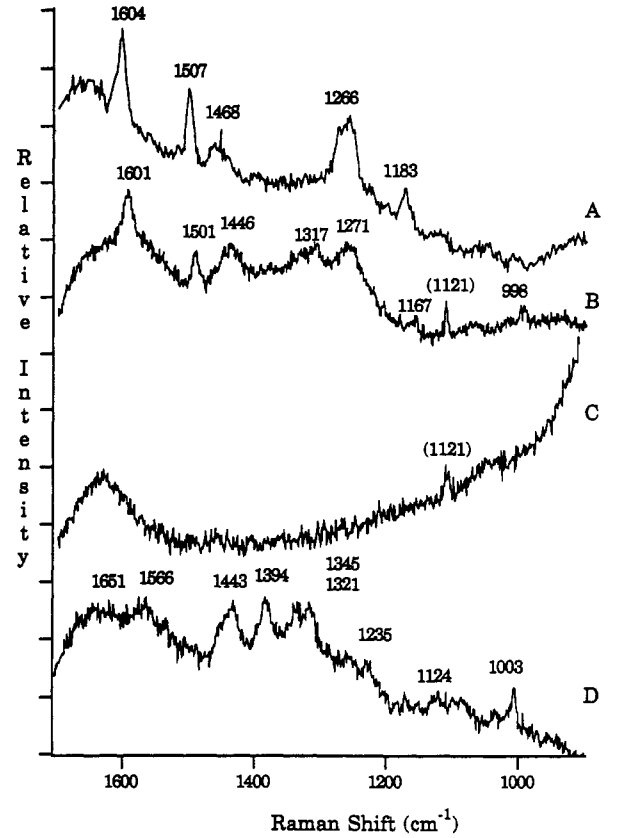
Preparation of Ag Colloids. Citrate-reduced Ag colloids were prepared by a modification of the Lee and Meisel method (Lee & Meisel, 1982). $AgNO_3$ (18 mg) was dissolved in 100 mL of filter-purified (Milli-Q) water, and the solution was brought to a boil. To this was added 2 mL of 1% sodium citrate, and the solution was allowed to boil for approximately 20 min more. The pH of the colloid at room temperature, which was typically between 8.5 and 10 after this procedure, was adjusted to the desired value (7.5–8.0) with 0.1 M HCl.

Preparation of SERRS Samples. Pseudomonas putida CCD was prepared as described previously (Broderick & O'Halloran, 1991). SERRS samples were prepared by adding 10 μL of 1 M NaCl to 200 μL of Ag colloid. Within 5 s the color of the colloid changed from yellow to gray, indicating the formation of colloidal aggregates. Immediately following this color change, a given amount of protein was added. After mixing, 10 μL of 0.5% melted agarose gel was added and mixed into the colloid suspension. The resulting SERRS sample is a viscous liquid rather than a solid gel.

RRS and SERRS. All RRS and SERRS experiments reported herein were performed using the 514.5-nm line of a Spectra Physics model 171-18 argon ion laser or the 647.1-nm line of a Coherent model CR-500K krypton ion laser. Raman scattered radiation was detected using a SPEX Model 1401-II double-grating monochromator equipped with a cooled RCA C31034A-02 photomultiplier tube, photon counting detection electronics, and an IBM PC-XT based data acquisition system. Vibrational bands in all spectra are reported relative to the 992-cm^{-1} band of benzene which is used as an *ex situ* intensity standard. All samples were rotated at ca. 60 rpm, and temperature was controlled to ±1 K in a stream of cooled N_2(g).

RESULTS AND DISCUSSION

RRS of CCD in Solution. CCD, like other intradiol dioxygenases (Felton et al., 1978; Tatsuno et al., 1978; Keyes et al., 1979; Que & Herstand, 1979), exhibits a broad visible absorption band resulting from tyrosinate to Fe(III) LMCT transitions (Figure 1b) (Broderick & O'Halloran, 1991). The RR spectrum of a 1.6 mM CCD solution obtained with λ_{ex} = 647.1 nm is shown in Figure 1a, spectrum A. This spectrum shows the characteristic tyrosinate ring modes of CCD. The signal-to-noise ratio (S/N) of RR scattering from CCD obtained with λ_{ex} = 647.1 nm was superior to that obtained with λ_{ex} = 514.5 nm in spite of the fact that the molar extinction coefficient, ϵ, at 647.1 nm is only $\sim$1000 M^{-1} cm^{-1} compared to ϵ = $\sim$2900 M^{-1} cm^{-1} at 514.5 nm. The lower S/N at λ_{ex}



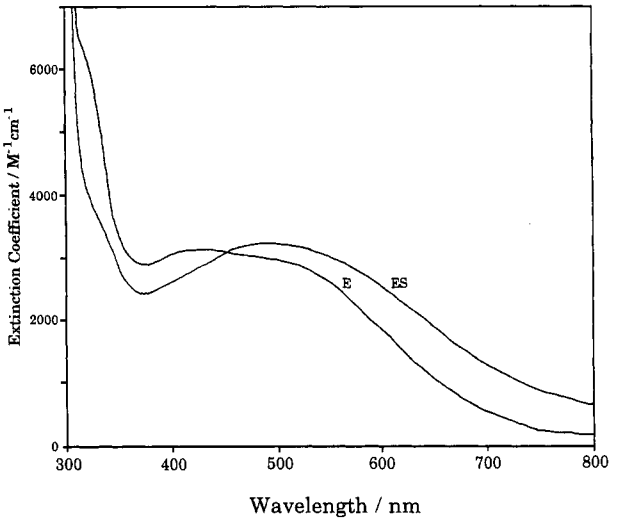


FIGURE 1: (a, top) RRS and SERRS of chlorocatechol dioxygenase. (A) Solution RR spectrum of 1.6 mM CCD: 65 mW of λ_{ex} = 647.1 nm, scan rate = 0.45 cm^{-1} s^{-1}, integration time = 5 s, bandpass = 3.9 cm^{-1}. (B) SERR spectrum of 13 μM CCD adsorbed on aggregated Ag colloid: 40 mW of λ_{ex} = 514.5 nm, scan rate = 1.8 cm^{-1} s^{-1}, integration time = 2 s, bandpass = 4.2 cm^{-1}. (C) Solution RR spectrum of 13 μM CCD: 40 mW of λ_{ex} = 514.5 nm, scanning parameters same as spectrum B. (D) SER spectrum of 10 μM apo-CCD adsorbed on aggregated Ag colloid: 40 mW of λ_{ex} = 514.5 nm, scanning parameters same as spectra B. For spectra A, B, C, and D, S/N was improved by coaddition of three identical scans, T = 20 °C, and solution conditions are 20 mM Tris-HCl at pH 7.8. The Raman shifts of the peaks are listed in Table I. The feature at 1121 cm^{-1} is due to Hg emission from room lights. The tick marks on the relative intensity scale correspond to 1500 counts s^{-1} for spectrum A and 500 counts s^{-1} for spectra B, C, and D. (b, bottom) Electronic absorption spectrum of chlorocatechol dioxygenase. The electronic absorption spectrum of native CCD was recorded at T = 20 °C on a 0.13 mM solution in 37 mM Tris-HCl/50 mM NaCl buffer at pH 7.6. The electronic absorption spectrum of the ES complex of CCD was recorded as above but under nitrogen in the presence of 3.0 mM catechol substrate.

Table I: SERRS Vibrational Modes and Assignments for Chlorocatechol Dioxygenase

CCD RRS	native CCD SERRS	apo-CCD SERS	assignment[a]
		755 (w)	Trp
792 (w)[b]			
	998 (m)	1003 (m)	Phe
		1124 (w)	Gly
1183 (w)	1167 (m)		Tyr C–H be[c]
		1235 (w)	Trp
1266 (m)	1271 (m)		Tyr C–O str[c]
	1317 (m)	1321 (m)	Tyr
		1345 (m)	Trp
		1394 (m)	COO–
1468 (w)	1446 (m)	1443 (m)	C–H be[d]
1507 (m)	1501 (m)		Tyr C–C str[c]
		1566 (w)	Phe, Tyr, Trp
1604 (m)	1601 (s)		Tyr C–C str[c]
		1651 (w)	

[a] Possible assignment of vibrations to amino acid side chains, based on previous SERS studies on amino acids and small peptides (Nabiev & Savchenko, 1983; Kim et al., 1987; Lee, H. I., et al., 1988; Chumanov et al., 1990; Herne et al., 1991). [b] Relative intensities: w = weak; m = medium; s = strong. [c] Assignments of the typical catechol dioxygenase vibrations based on model complex studies (Tomimatsu et al., 1976); be = bend; str = stretch. [d] Attributed to the protein backbone.

= 514.5 nm is a consequence of the fact that the larger signal due to RR enhancement was more than offset by a far larger increase in noise resulting from intrinsic CCD fluorescence. In fact, no vibrational bands have been observed for native CCD in the absence of substrate with λ_{ex} = 514.5 nm.

SERRS and Activity of CCD Adsorbed on Silver Colloid. Figure 1a, spectrum B shows the SERRS spectrum of 1.9 μM CCD adsorbed on aggregated Ag colloid, the pH of which had been adjusted to between 7.5–8.0 prior to addition of NaCl or protein. The frequencies of the observed vibrational bands in this spectrum are very similar to those in the solution RR spectrum of CCD, indicating that the active site has not been drastically altered by adsorption. In addition, adsorption to the silver surface greatly decreases the intrinsic fluorescence of the protein, thus allowing us to obtain the SERRS spectrum with λ_{ex} = 514.5 nm. The intensity of the SERRS signals are enhanced by a factor of 10^3 as compared to the solution RR spectrum when intensity units are expressed as counts s^{-1} W^{-1} $molecule^{-1}$. A solution RR spectrum of CCD at 1.9 μM is shown in Figure 1a, spectrum C, demonstrating that, at the same concentration of enzyme that was used in the SERRS spectrum, no solution RR spectrum is observed. Adsorption of apo-CCD to aggregated Ag colloid and excited at λ_{ex} = 514.5 nm results in the spectrum shown in Figure 1a, spectrum D. This spectrum is quite similar to that observed for adsorbed native CCD, except that the tyrosinate vibrations at 1601, 1501, 1271, and 1167 cm^{-1} are not observed. This supports our conclusion that the SERRS spectrum of native CCD results from resonance-enhanced as well as surface-enhanced vibrations. The vibrational bands observed for the various samples and their assignments are listed in Table I. The similarity of the SERR spectrum of native CCD to the solution RR spectrum suggests that the active site structure is conserved, as both show vibrational features typical for iron–tyrosinate proteins. This similarity also demonstrates that SERRS, like RRS, can be a generally useful tool for structural studies of metalloprotein active sites.

The RR spectrum of CCD (Figure 1a, spectrum A) is that of enzymatically active protein. Under the conditions of the SERR spectrum B and in the presence of Ag colloid, the CCD activity is 60–85% of the activity of the same concentration of CCD in the absence of Ag colloid. To test whether the

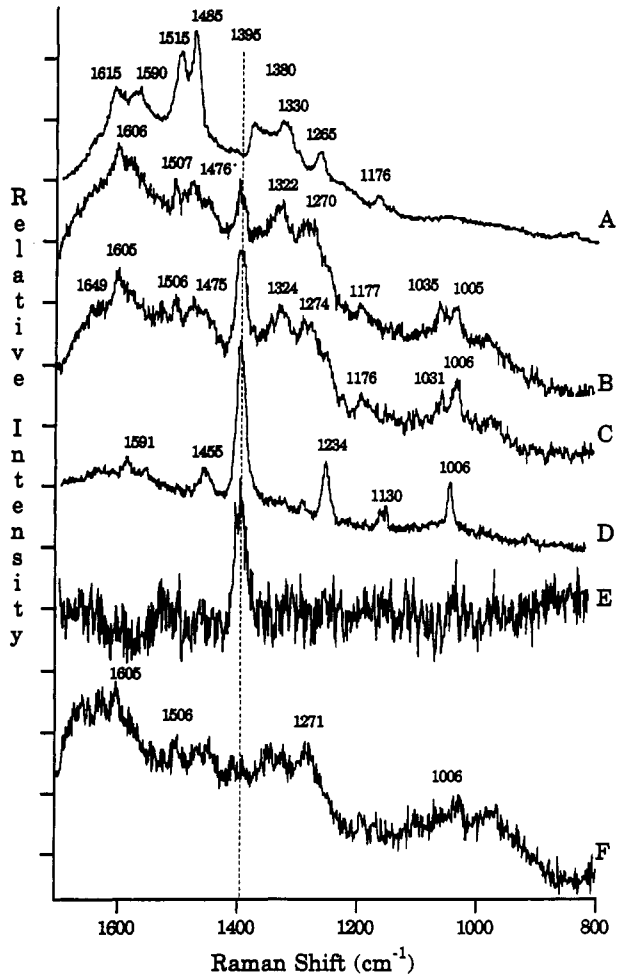


FIGURE 2: SERRS of catechol turnover at the silver surface. (A) SER spectrum of 22 μM catechol: 40 mW of λ_{ex} = 514.5 nm, scan rate = 1.8 cm^{-1} s^{-1}, integration time = 2 s, bandpass = 4.2 cm^{-1}, T = 20 °C, solution conditions = 20 mM Tris-HCl at pH 7.8. (B) SERR spectrum of anaerobic 13 μM CCD–catechol. (C) SERR spectrum of a sample in spectrum B after exposure to oxygen. (D) SER spectrum of 22 μM 2-chloro-*cis,cis*-muconate. (E) SERR difference spectrum of C – B. (F) SERR spectrum observed when both CCD and *cis,cis*-muconate are present at 13 μM. λ_{ex}, scanning parameters, T, and solution conditions same as spectrum A for spectra B–F. The tick marks on the relative intensity scale correspond to approximately 5000 counts s^{-1} for spectrum A, 2090 counts s^{-1} for spectrum D, and 1000 counts s^{-1} for spectra B, C, E, and F.

SERRS-active species is also the enzymatically active species, we have obtained the SERR spectra of anaerobic, Ag-adsorbed CCD–catechol complexes before and after exposure to O_2. Figure 2A shows the SER spectrum of catechol at 18 μM. Figure 2B shows the spectrum obtained when anaerobic CCD–catechol (1:1) complex is adsorbed to the silver colloid at a final enzyme concentration of 13 μM. The formation of the enzyme–substrate complex was verified in solution by the color change from pink to blue-purple upon addition of S to E (see Figure 1b). The ES complex was then added to the anaerobic aggregated silver colloid. The SERR spectrum of the anaerobic ES complex is different from the surface-enhanced spectra of either E or S alone and thus most likely results from the ES complex bound to the silver surface. This is further verified by the spectral changes upon oxygen addition. Upon exposure of the colloid-adsorbed ES complex to O_2, the spectrum changes to that shown in Figure 2C. The sharp band that appears at 1395 cm^{-1}, which can be attributed to a carboxylate vibrational mode, is indicative of product

Table II: Raman Shifts for the Spectra in Figure 2[a]

S	ES	ES + O_2	P	(ES + O_2) – ES	EP
		1649 (w)[b]	1631 (w)		
1615 (w)					
	1606 (m)	1605 (m)			1605 (w)
1590 (w)			1591 (w)		
		1535 (w)	1557 (w)		
1515 (s)					
	1507 (w)	1506 (w)			1506 (w)
1485 (s)	1476 (w)	1475 (w)	1455 (m)		
1380 (m)	1396 (m)	1395 (s)	1392 (s)	1395 (s)	
1330 (m)	1322 (m)	1324 (m)			
1265 (w)	1270 (m)	1274 (m)	1279 (w)		1271 (w)
			1234 (m)		
1176 (w)	1177 (w)	1176 (w)			
			1130 (w)		
	1035 (w)	1031 (w)			
	1005 (w)	1006 (w)	1006 (m)		1006 (w)

[a] The six columns correspond to the spectra A–F in Figure 2. [b] Relative intensities: w = weak; m = medium; s = strong.

formation, as can be seen by comparison to the SER spectrum of the product *cis,cis*-muconate in Figure 2D. The difference spectrum ES(aerobic) – ES(anaerobic) (Figure 2E) shows that the 1395-cm^{-1} band in the ES complex has increased in intensity after exposure to O_2. The presence of this band in Figure 2B may indicate that some substrate turnover had occurred prior to intentional exposure to oxygen, possibly due to a small amount of oxygen contaminating the "anaerobic" sample. This observation of substrate turnover at the silver surface strongly suggests that the SERRS-active species is also enzymatically active.

Further evidence that the observed turnover of substrate is due to Ag-adsorbed enzyme is obtained by addition of the EP complex to Ag colloid. A spectrum (Figure 2F) similar to that of the native enzyme (Figure 1B), but not to that of the EP complex (Figure 2C), is observed. In particular, the 1395-cm^{-1} feature is absent in Figure 2F. This enzyme and product solution was made prior to adsorption on the colloid by allowing the 1:1 complex of CCD–catechol to turn over in oxygenated solution for a period of time 600-fold longer than that required for complete turnover. Consumption of the ES complex is verified by the color change from bluish-purple to red. Stopped-flow spectroscopic studies of turnover by CCD have shown that, at concentrations of 50–80 μM in E and P, the EP complex dissociates quickly into free E and free P (Broderick et al., 1993). Thus, in the SERRS sample, in which [E] = [P] = 13 μM, the CCD and *cis,cis*-muconate are expected to be present as the free species rather than as an EP complex. Since we do not observe vibrational bands due to product in this sample shown in Figure 2F, we conclude that the presence of CCD prevents SERRS vibrational enhancement of the free product. This E + P SERR spectrum demonstrates (1) that EP in solution at these concentrations does not adsorb to the Ag surface as a complex, but rather dissociates, allowing uncomplexed E to adsorb and produce a native enzyme spectrum, and (2) that, in the presence of native enzyme, free product is prevented from adsorbing to the colloid surface. Thus the SERR spectrum shown in Figure 2C, which shows vibrational bands attributable both to enzyme and to *cis,cis*-muconate, must be a spectrum of the EP complex adsorbed to the silver surface, as free P would not produce vibrational bands in the presence of free E. Because the EP complex in solution is known to dissociate rapidly, this SERRS-active EP complex is most likely to have been formed at the silver surface with P "trapped" in the active site to prevent dissociation. These results indicate that turnover is occurring

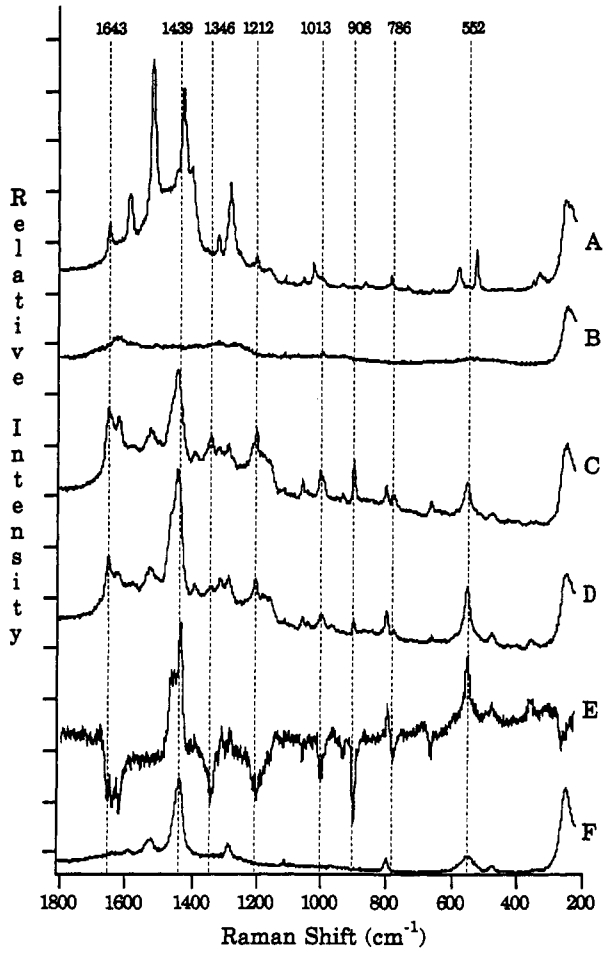


FIGURE 3: SERRS of the CCD–tetrachlorocatechol complex. (A) SER spectrum of <90 μM TCC: 10 mW of λ_{ex} = 514.5 nm, scan rate = 1.8 cm^{-1} s^{-1}, integration time = 2 s, bandpass = 4.2 cm^{-1}, T = 20 °C, solution conditions = 20 mM Tris-HCl at pH 7.8. (B) SERR spectrum of 12 μM CCD. (C) SERR spectrum of the anaerobic CCD–TCC complex. (D) SERR spectrum of the CCD–TCC complex 9 h after the exposure to oxygen. (E) SERR difference spectrum of C – D. (F) SERR spectrum of the CCD–tetrachloromuconate complex. λ_{ex}, scanning parameters, T, and solution conditions same as spectrum A for spectra B–F. [CCD–TCC] = 12 μM for spectra C, D, E, and F. The tick marks on the relative intensity scale correspond to approximately 1000 counts s^{-1} for spectra A–D and F and 333 counts s^{-1} for spectrum E.

at the silver surface and that the SERRS active species is also the enzymatically active species.

SERRS of the CCD–Tetrachlorocatechol (TCC) Complex. The SERR spectrum of the anaerobic CCD–TCC complex is shown in Figure 3C, with the SER spectrum of the same concentration of tetrachlorocatechol or enzyme alone shown in Figure 3 spectra A and B, respectively, for comparison. The low solubility of TCC makes it difficult to establish the exact concentration in these solutions. However, we estimate [TCC] < 90 μM. The spectrum in Figure 3C is clearly a spectrum of the EI complex and not of uncomplexed E or I bound at the surface. The binding of tetrachlorocatechol to the enzyme results in a large increase in the intensity of the observed Raman bands, relative to those of the enzyme complex with catechol. In addition, the surface enhancement results in the appearance of many additional bands when compared to the solution RR spectrum of the CCD–TCC complex (J. B. Broderick and T. V. O'Halloran, unpublished results). Upon exposure of the complex in Figure 3C to dioxygen, the spectrum changes slowly. The spectrum taken approximately 9 h after exposure to dioxygen shown in Figure 3D reveals further

Table III: Raman Shifts for Spectra in Figure 3

TCC[a]	E[b]	E·TCC[c]	E·TCC + O_2[d]	diff[e]	TCM[f]
237 (m)[g]		228 (m)	227 (m)	237 (–)[h]	234 (s)
329 (w)			338 (w)	338 (+)	
		470 (w)	472 (w)	472 (+)	472 (w)
525 (m)					
		553 (m)	550 (m)	550 (+)	552 (m)
582 (w)					
		667 (w)	667 (w)	667 (–)	
798 (w)		786 (w)	787 (w)	786 (–)	
		811 (w)	808 (m)	808 (+)	811 (m)
		910 (m)	908 (w)	908 (–)	
	1004 (w)	1013 (m)	1009 (w)	1013 (–)	
1037 (w)		1068 (m)	1068 (w)	1068 (–)	
1178 (w)		1190 (m)	1188 (m)	1190 (–)	
1212 (w)		1210 (m)	1212 (m)	1212 (–)	
1291 (m)	1275 (w)	1293 (w)	1292 (w)		1292 (w)
1326 (w)	1324 (w)	1347 (m)	1346 (w)	1346 (–)	
1405 (w)	1393 (w)	1397 (m)	1397 (w)		
1428 (s)		1444 (s)	1444 (s)	1441 (+)	1439 (s)
				1453 (+)	
1516 (s)	1505 (w)	1522 (m)	1520 (m)		1515 (m)
1580 (m)					
	1613 (w)	1611 (m)	1614 (w)	1613 (–)	
				1628 (–)	
1639 (m)		1640 (m)	1639 (m)	1643 (–)	

[a] TCC = tetrachlorocatechol; Figure 3A. [b] E = native enzyme; Figure 3B. [c] E·TCC = anaerobic enzyme–tetrachlorocatechol complex; Figure 3C. [d] Enzyme–tetrachlorocatechol complex 9 h after addition of oxygen; Figure 3D. [e] diff = the difference spectrum in Figure 3E. [f] TCM = tetrachloromuconate, the product of the turnover of tetrachlorocatechol by CCD; Figure 3F. [g] Relative intensities: w = weak; m = medium; s = strong. [h] For peaks in the difference spectrum, (–) denotes a negative peak, and (+) denotes a positive peak.

turnover as does the difference spectrum shown in Figure 3E. If the CCD–TCC complex is allowed to turn over in air in the absence of colloid for a prolonged period and then used in preparation of the SERRS sample, the spectrum in Figure 3F is obtained. As the positive peaks in the difference spectrum (Figure 3E) resemble those of the EP complex in Figure 3F, turnover of TCC by CCD appears to be occurring upon exposure of the adsorbed CCD–TCC complex to dioxygen. The slow change of the CCD–TCC SERR spectrum after exposure to dioxygen and the tight binding of this tetrahalogenated product are consistent with the very slow rate of turnover of TCC by CCD (Broderick & O'Halloran, 1991).

CONCLUSIONS

SERRS has been used for the first time to study a non-heme metalloprotein. We demonstrate that the enzyme, CCD, adsorbed on a SERS-active Ag surface can retain both its native conformation and its biological activity by directly observing the SERRS spectra of its complexes with substrate, inhibitor, and product during catalytic turnover. These results represent an advance in the development of SERRS as a structural probe in the study of biological molecules.

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