In CBD derivatives like **3** the barrier to pseudorotation to an equivalent rectangular geometry via a square transition state should therefore be reduced from the roughly 10 kcal/mol that the best ab initio calculations find for the parent molecule.[16] This may explain the absence of line broadening in the ^{13}C NMR spectrum of the tri-*tert*-butyl derivative of CBD at even very low temperatures.[17]

In summary, our calculations show relatively small energy increases for large increases in the lengths of the double bonds in CBD. The energy increases are minimized if, concomitant with double bond lengthening, single bond shortening occurs. These theoretical findings provide an explanation of the dramatic changes in geometry with increasing steric interactions between substituents on the double bonds in the series **1-3**.[18]

Acknowledgment. Acknowledgement is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this work. We also thank the National Science Foundation and the Alfred P. Sloan Foundation for financial support.

(16) The data in Table I show the fraction of the barrier that is eliminated after partial distortion of CBD from the rectangular minimum toward a square geometry.

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(18) It should be noted that the geometry and apparent diamagnetic nature of **3** provide evidence for the correctness of the theoretical prediction of a singlet ground state for CBD, even at a square geometry.[6-8,14b,19]

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Surface-Enhanced Resonance Raman Scattering from Cytochrome *c* and Myoglobin Adsorbed on a Silver Electrode

Sir:

The study of adsorbed molecules on metal surfaces by Raman spectroscopy, once an almost impossible task, has rapidly developed into a major area of interest in recent years. The activity in this field was kindled by the original observation of Raman spectra of pyridine on Ag in an electrochemical cell[1] and the discovery that these spectra were observed only because of the previously unrecognized enhancement of the Raman scattering cross section (approximately 10^6) by the silver electrode surface.[2] Such spectra have now come to be known as surface-enhanced Raman Spectra (SERS).[3] SERS has been observed for a variety of small neutral molecules (e.g., *N*-heterocycles, aromatic and aliphatic amines,[3] olefins,[4a] CO[4b,c]) and ions (e.g., CN^-,[5a-d] SCN^-,[5e-g] N_3^-, $Pt(CN)_4^{2-}$, $Ru(CN)_6^{-4}$, MoO_4^{-2},[6]) on silver and copper electrodes.[6] In addition, Pettinger has observed SERS for the Au/pyridine system.[7] In all of the above systems, the laser excitation wavelengths used to obtain the SERS spectra were far from resonance with an electronic transition localized in the adsorbate. If, on the other hand, the laser excitation wavelength is coincident with an absorption band in the adsorbate, the resonance Raman (RR) and SER enhancements are multiplicative. Jeanmaire and Van Duyne[2,3] were the first to exploit the enormous enhancement possible under these circumstances when they obtained the combined RR and SER spectrum of crystal violet on both a silver thin film and a bulk polycrystalline electrode using only a few milliwatts of laser power. Since then, however, there have been relatively few adsorbates studied which exhibit this type of combined enhancement.[8]

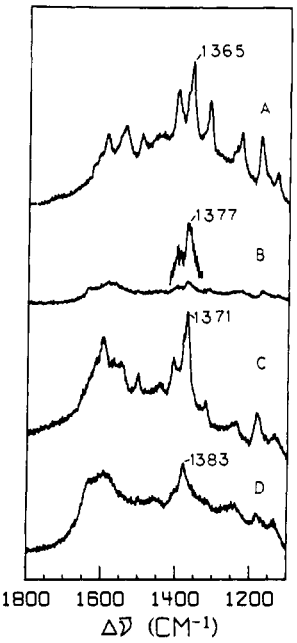

Figure 1. Surface-enhanced RR spectra of 1×10^{-6} M sperm whale myoglobin and tuna cytochrome *c* adsorbed on an anodized Ag electrode. (A) Cytochrome *c* at −0.6 V vs. SCE; (B) cytochrome *c* at −0.2 V vs. SCE; (C) myoglobin at −0.6 V vs. SCE; (D) myoglobin at −0.2 V vs. SCE. The excitation wavelength was 514.5 nm and the power was 40 mW. Scan parameters are as follows: slit width = 2 cm^{-1}, scan rate = 0.3333 Å/s, and 1.0-s counting interval. For simplicity, only those bands discussed in the text are labeled.

Although the mechanism of the SERS enhancement is not yet completely understood, much progress has been made toward the development of such understanding.[9] One of our research goals has been to pursue the practical applications of the SERS effect which are independent of its mechanism in parallel with studies directed at the origin of the enhancement. In the biological area, there are numerous possible applications of a practical nature which can be approached with SERS + RRS. One example is to utilize the enhanced sensitivity to determine the Raman or RR spectrum of scarce materials. A second possibility is to use SERS to monitor electron-transfer reactions of redox-active proteins or membrane preparations adsorbed on an electrode. Spontaneous protein adsorption at electrodes, an annoyance in the past, should prove advantageous for this purpose. In the results reported here,

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Table I. Comparison of Oxidation and Spin-State Marker Bands of Cyt c in Solution and at a Ag Electrode[a]

ferriCyt c[b] (low spin)	Cyt c at Ag electrode, −0.2 V	ferroCyt c[c] (low spin)	Cyt c at Ag electrode, −0.6 V
1636	1638	1622	1625
1582	1588	1584	1589
1502	1502	1493	1498
1376	1377	1362	1365

[a] Frequencies are in cm^{-1}. [b] From ref 12, 13, 15, and this work. [c] From ref 12, 13, and 15.

Table II. Comparison of Oxidation and Spin-State Markers of Mb in Solution and at a Ag Electrode[a]

ferriMb[b] (low spin)	Mb at Ag electrode, −0.2 V	ferroMb[b] (low spin)	Mb at Ag electrode, −0.6 V
1644	1635 (shoulder)	1638	1640
1561	1560	1562	1569
		1544	1547
1507	1501	1503	1502
1374	1383	1375	1371

[a] Frequencies are in cm^{-1}. [b] From ref 12 and 14.

we show that both of these potential applications can be, in fact, realized. The combination of SER and RR enhancement has permitted us to observe the vibrational spectrum of the heme chromophore in highly dilute solutions of both myoglobin (Mb) and cytochrome c (Cyt c). These two proteins were chosen for the SERS + RRS experiments because both are structurally well characterized and their conventional RR spectra have been extensively studied as a function of central metal oxidation state, spin state, environment, and laser excitation wavelengths. To our knowledge, this is the first time SERS has been applied to complex biomolecules.[10]

Stock solutions of tuna Cyt c and sperm whale Mb were prepared at 1×10^{-3} M concentration in distilled water. Aliquots of these solutions were injected into the electrochemical cell, which contained degassed 0.1 M Na_2SO_4 in distilled and deionized water (Milli-Q, Millipore Corp.). The Raman apparatus, electrochemical cell, potentiostat, and electrode preparation have all been described.[2] The anodization procedure consisted of a single oxidation and reduction cycle. Oxidation of the Ag electrode was achieved at +0.45 V vs. SCE, followed by reduction at −0.6 V. Charge passed in the oxidation step was equivalent to 25 mC/cm^2. Electrode surface characterization by scanning electron microscopy has shown recently[11] that this anodization step produces regular, uniformly distributed spherical particles of Ag ≥ 250–2000 Å in diameter. The Ag surface is also cleaner after the anodization on the basis of Auger electron spectroscopic measurements made before and after electrochemical pretreatment.[11a]

The surface-enhanced RR spectrum of Cyt c on a Ag electrode is shown in Figure 1 (A and B) at two different potentials using 514.5-nm excitation. The spectrum observed at −0.6 V vs. SCE exhibited very high signal-to-noise ratios and is characteristic of the heme chromophore, i.e., no protein vibrations are enhanced under these conditions. Moreover, no RR spectra could be observed for the solution species at this concentration (1×10^{-6} M) and excitation wavelength. Excellent surface spectra were obtained at this potential for solution concentrations as low as 100 nM. Though more detailed studies are required to produce an accurate measurement of the surface enhancement factor, a semiquantitative estimate can be provided by comparing the intensity of the 1365-cm^{-1} band of Cyt c on the electrode with that observed for an equal number of Cyt c scatters in solution under the same experimental conditions. When monolayer coverage at the electrode is assumed, the surface signal is approximately 5 orders of magnitude greater than that in solution! This enhancement is, of course, in addition to that produced by the resonance effect.

The positions of the spin- and oxidation-state marker bands of Cyt c on the electrode surface indicate that the heme Fe is in its low-spin, reduced state at −0.6 V.[12,13] This result implies that the Fe is reduced at the electrode, since it is present as Fe(III) in the bulk Cyt c solution. Stepping the electrode potential to a more positive value, −0.2 V, results in the surface spectrum shown in Figure 1B. It is much less intense than that at the negative potential, as emphasized in the figure, where both spectra are presented on the same ordinate scale. Depolarization ratios for all bands at both potentials are greater than 0.5. When the −0.2-V spectrum is displayed on an expanded scale (the insert depicts the oxidation-state marker), the band positions can be determined accurately and are indicative of low-spin Fe(III). Hence, the surface-bound Cyt c is reoxidized at this potential. Preliminary cyclic voltammetry measurements on the anodized Ag electrode verify that reduction and oxidation of the surface Cyt c does occur, but the electron-transfer kinetics are slow. Table I lists the band positions for the oxidation and spin-state markers of ferri- and ferroCyt c in solution together with the respective values for the protein adsorbed on the Ag electrode at −0.2 V and −0.6 V. In all cases, the frequencies of the adsorbed Cyt c are within 6 cm^{-1} of those for the solution species.

The surface-enhanced RR spectrum of Mb on a Ag electrode is shown in Figure 1C,D for two different electrode potentials. As was noted in the case of Cyt c, the spectrum is most intense at −0.6 V. All bands exhibit depolarization ratios greater than 0.5 at both potentials. Table II compares the oxidation- and spin-state markers for low-spin Mb in its oxidized and reduced state[12,14] with the values observed for Mb at the electrode surface for the potentials −0.2 and −0.6 V, respectively. The correspondence between the surface Mb and solution Mb spectra is best if values for low-spin Mb are used in the comparison. However, Mb in solution is present in the high-spin aquamet Fe(III) state, which suggests the Mb undergoes a change in spin state at the electrode. Another possibility is that the Mb is in its high-spin state, but the frequencies are shifted due to direct interaction of the heme group with the electrode. In any case, a shift in the oxidation-state marker band at 1371 cm^{-1} in the −0.6-V spectrum to 1383 cm^{-1} in the −0.2-V spectrum implies that oxidation of Mb occurs at the electrode surface. There are two possible oxidation reactions to consider. First, the solution Mb may undergo reduction to the Fe(II) state at −0.6 V and then reoxidation to Fe(III) at −0.2 V. Alternatively, if Mb is not reduced at −0.6 V from Fe(III) as is present in solution, it is conceivable that oxidation to Fe(IV)might occur at −0.2 V. The oxidation-state marker for Fe(IV) is found near 1383 cm^{-1}.[15] However, this latter possibility seems unlikely in veiw of the fact the $E°$ for Fe(III) reduction to Fe(II) is −0.290 V vs. SCE.[16] Hence, it is more likely that reduction of Fe(III) to Fe(II) occurs at −0.6 V for Mb adsorbed at the electrode followed by its reoxidation at −0.2 V.

The nature of the Cyt c and Mb interactions with the Ag electrode surface cannot be specified with certainty at the present time. It is conceivable that the heme chromophore interacts directly with the electrode, either as a result of its detachment from the protein matrix or due to denaturation of the protein. Cyt c would not be as likely to release its heme at the surface, since it is covalently bound to the protein in contrast to the more labile coordination and hydrogen-bonding interactions which attach the heme to the apoprotein of Mb. Extensive denaturation of Cyt

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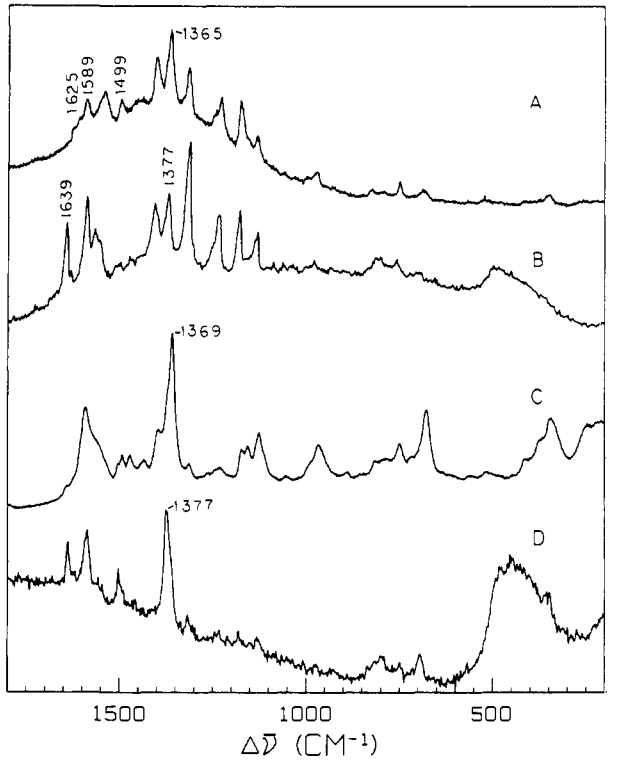

Figure 2. Comparison of the surface-enhanced RR spectra of cytochrome *c* with that in solution. (A) Cytochrome *c* spontaneously adsorbed from a 1×10^{-6} M solution on a Ag electrode at −0.6 V vs. SCE—laser excitation wavelength was 514.5 nm; (B) 1×10^{-3} M solution of ferricytochrome *c*—laser excitation wavelength was 514.5 nm; (C) same as (A) except laser excitation wavelength was 457.9 nm; (D) same as (B) except laser excitation wavelength was 457.9 nm. The laser power in all of the above experiments was approximately 40 mW. The scan parameters were as follows: slit width = 5 cm^{-1} for solution and 2 cm^{-1} for surface spectra; scan rate = 0.20 Å/s for solution and 0.3333 Å/s for surface spectra. The broad feature between 350 and 500 cm^{-1} in (D) is due to Raman scatter from the glass sample tube. For simplicity, only those bands discussed in the text are labeled.

c at the electrode appears unlikely on the basis of the close correspondence of the surface and solution spectra. Also, the surface spectra were reproducible on repeated cycling of the electrode from negative to positive potentials, suggesting that little denatured and insoluble protein remained on the electrode at the positive potentials. In contrast, the possibility of heme loss or protein denaturation in the case of Mb appears more probable. Direct interaction of the heme with the electrode could explain possible spin-state changes and/or large differences in the marker-band frequencies in comparison to their values for Mb in solution. Shifts on the order of 10 cm^{-1} are usually encountered in the vibrational frequencies of small molecules adsorbed at Ag electrodes.[1-7] Further experimentation is needed to determine the possibility and extent of denaturation in both proteins.

Surface spectra of Cyt *c* which are obtained by using laser excitation wavelengths closer in resonance with the Soret transitions are even more intense than those observed by using 514.5-nm light. Figure 2 compares the surface (A) and solution (B) spectra of Cyt *c* resulting from 514.5-nm excitation with the respective surface (C) and solution (D) spectra resulting from 457.9-nm excitation. The improvement in the signal-to-noise ratio in the 457.9-nm surface spectrum (C) suggests that an even lower detection limit for surface adsorbed heme proteins may be achievable by using deep-blue excitation. For both excitation wavelengths, a comparison of the solution spectra with those at the surface at −0.6 V clearly shows a shift in the oxidation-state band from approximately 1365 to 1377 cm^{-1}. The data again suggest the surface-bound Cyt *c* is reduced at this potential.

It should be noted that some decrease in surface signal strength is observed for both proteins with prolonged irradiation. The loss in intensity does not appear to be caused by extensive photodegradation of the protein, since no new bands are produced in the RR spectrum. The original signal intensity can be regenerated in the dark at −0.6 V, which suggests that the signal decrease is caused by thermally induced protein desorption at the electrode.

It can be said with certainty that the RR spectra observed for Cyt *c* and Mb are of molecules adsorbed at the electrode surface and that the underlying surface enhancement has a basis similar to that for SERS of small molecules. The criteria on which this conclusion is based include the following: (1) the signal intensity is dependent upon anodization of the Ag electrode; (2) the signal intensity is potential dependent; (3) depolarization ratios for all of the Cyt *c* and Mb vibrations are near 0.5, in contrast to the range of values exhibited in solution (from 0.1 to ∞); (4) detectable enhancement of the RR spectra of these proteins could not be observed at a Pt electrode by using conventional Raman spectrometers. These observations will be further explored in a future publication.

In conclusion, the data presented, though of a preliminary nature, are highly encouraging with regard to possible applications of SERS to bioanalytical problems. Surface-enhanced RR spectra were obtained for highly dilute solutions of the heme-containing proteins Cyt *c* and Mb, indicating that SERS has potential for detecting RR scattering from extremely small amounts of biological materials. Besides the enhancement effect, the electrode may serve as a scavenger and concentrate the species of interest in the laser beam. The observation of redox changes in the heme group of these two proteins substantiates that it will be possible to study electron-transfer processes in biological systems at the electrode surface. Furthermore, the sensitivity of the RR spectrum of heme to its environment should enable a detailed investigation of the state of the protein at the electrode surface. This type of structural information is not available in conventional electrochemical experiments. With the development of methodology for preserving the native state of proteins at the electrode surface, the combination of SERS and RRS techniques may well prove to be one of the most sensitive and selective probes of biomolecular structure yet devised.

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Total Synthesis of Rifamycins. 1. Stereocontrolled Synthesis of the Aliphatic Building Block

Sir:

Rifamycins,[1,2] isolated from the fermentation medium of *Norcardia mediterranei* by Senti, Greco, and Ballotta in 1959,

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