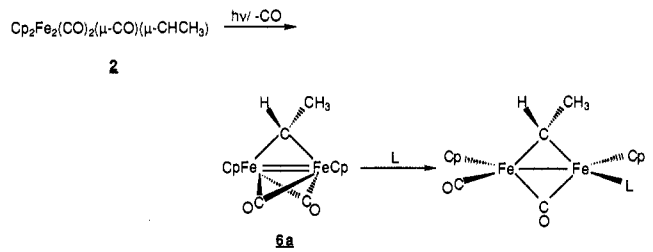
Scheme IV



bridged intermediate analogous to **2Z** is involved in the cis/trans isomerization of **2** and $Cp_2Ru_2(CO)_2(\mu\text{-}CO)(\mu\text{-}CMe_2)$; Bergman and Theopold favor a similar intermediate for the cis/trans isomerization in $Cp_2CoRh(CO)_2(\mu\text{-}CH_2)$.[8b,32a,51]

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While **2X** and **2Y** would be equally possible substitution intermediates, LFP studies support formation of a CO-loss species that we propose to be isostructural to **4**. Given the similarity in the electronic structures of **1** and **2**, we think it is quite plausible that the photochemistry of the two would be similar, except for the apparent absence of a radical pathway. Indeed, the dinuclear substitution photochemistry is similar, suggesting a mechanism for **2** entirely parallel to that for **1** (Scheme IV). The major difference between the two starting dimers is that the CO-loss product for **2** would possess a singlet ground state. The implication of the difference in the spin state of the intermediate is currently under investigation.

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Surface-Enhanced Resonance Raman Scattering from Cytochromes P-450 Adsorbed on Citrate-Reduced Silver Sols

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Abstract: Four mammalian liver microsomal cytochrome P-450 enzymes were adsorbed onto citrate-reduced silver colloidal particles, and the active site of each was studied by surface-enhanced resonance Raman scattering (SERRS) spectroscopy. There were differences in the spin states of the heme groups in each protein. Methylcholanthrene-induced rat liver cytochrome P-450 IA2 was approximately 90% high spin. Phenobarbital-induced rat liver cytochromes P-450 IIB1 (90% low spin) and P-450 IIB2 (mixed high and low spin) and rabbit liver cytochrome P-450 IIB4 (100% low spin) were shown to be biologically active while adsorbed on the metal surface by following benzphetamine-induced low- to high-spin-state conversion of the heme prosthetic group. On formation of the mixed low- and high-spin-state P-450 IIB4 benzphetamine complex, the low-spin-state marker band ν_{10} shifts down by 3 cm^{-1}. It is suggested that reorganization of the water molecules in the pocket upon binding of the benzphetamine is responsible. P-450 proteins are readily denatured at a sol surface, and the protein/sol preparation described in this paper is carefully controlled so that the surface of the sol is "biocompatible". The ability of this particular sol to support an active enzyme is due to a layer of citrate ions that form a coating on the sol surface of the enzyme, providing a spacer between the silver and the protein and protecting it from silver-induced reactions. In addition, the heme group is not on the surface of the protein. This fact and the effect of the citrate layer are sufficient to preserve the integrity of the active site, and yet the distance between the silver and the heme is close enough for SERRS. If the sol is carefully prepared, no denaturation involving changing spin states is observed, although denaturation to P-420 can be forced with acid or alkali. Surface selection rules suggest that the heme orientation is less flat relative to the surface of the metal particle than is heme in cytochrome *c*. Thus, a "biocompatible sol" has been prepared and reactions of the immobilized enzymes at the metal surface have been examined in situ in contact with aqueous media by using only a few nanograms of sample.

Introduction

The intensity amplification gained from specially prepared silver surfaces along with the inherent selectivity of the chromophores associated with natural pigments enables surface-enhanced resonance Raman scattering (SERRS) spectroscopy[1] to provide information on the orientation, adsorption, and activity of adsorbed enzymes.[2] Since very small amounts of protein can be observed by SERRS and since contact can be maintained between the protein adsorbed onto the particle surface and the aqueous media or buffer, reactions of adsorbed proteins with substrates can be probed in situ. Thus, the SERRS technique could be of considerable value in the study of reactions of immobilized proteins,

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provided the surface of the sol particle is modified to be "biocompatible". Therefore, provided that the integrity of the proteins can be retained and the chemistry of the surface controlled so that it is biocompatible, SERRS will enable in situ studies of adsorption and protein–protein interactions necessary for an understanding of systems such as the mitochondrial respiratory electron transport chain and the microsomal-bound monooxygenase system associated with bilayer lipid membranes in vivo.[3,4] In this study, liver microsomal cytochrome P-450 monooxygenase enzymes,[5] which catalyze the insertion of oxygen into a substrate in vivo,[6] are immobilized on a specially prepared stable silver colloid. In addition to its use as a lead system for the study suggested above, SERRS may also be of value in studies of molecular recognition[7a] and enzyme reactors.[7b,c] For example, there has been one recent suggestion of the use of regiospecific hydroxylation of bicyclic molecules by mutagenically altered cytochrome $P\text{-}450_{cam}$.[8]

Substrate-induced low- to high-spin-state conversion of the prosthetic heme of cytochrome P-450 often characterizes the enzyme–substrate interaction.[9,10] In the microsome, the charged nature of the head groups of the phospholipids constituting the membrane exerts an additional electrostatic influence, which may be responsible for the low- to high-spin-state conversion detected by electronic spectroscopy when cytochrome P-450 is reconstituted in lipid vesicles.[11] Microsomal cytochrome P-450 exists in multiple forms with closely related structures (in mammals the cytochrome P-450 family comprises at least 50 genes[12]), and the preservation of differences between various structurally related isozymes and disparate forms may be used as confirmation that colloid-induced "denaturation" has been avoided, or at least minimized.

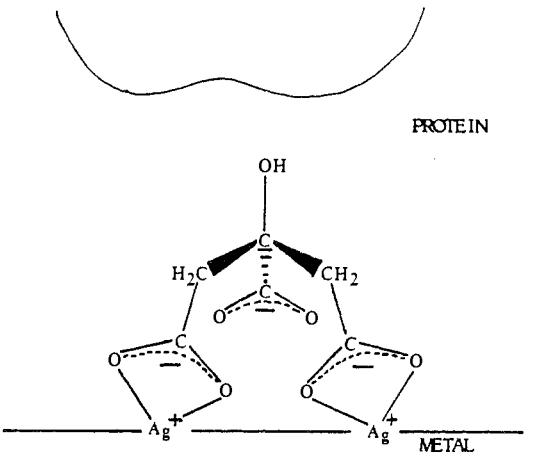

Figure 1. Representation of negatively charged citrate molecules adsorbed on a positively charged silver surface (solid line).

One difference between cytochromes P-450 that is sensitive to changes in conformation and enzymatic activity is the heme spin state,[13] a characteristic which is particularly amenable to study with resonance Raman spectroscopy.[14] The correlation of resonance Raman spectra with the spin state of various heme proteins and model complexes is well documented.[15] Differences in the spin states of the hemes of different cytochromes P-450 must be conserved, if the same differences in biological activity observed in solution are to be retained upon adsorption onto the silver sol. Further confirmation of biological activity is available from studying the effect on heme spin state of formation of the cytochrome P-450–substrate complex. For example, if it is known from solution studies that addition of substrate induces a spin-state change on the heme group, then this should be reflected in the SERRS spectra from the colloid.

Our preferred "SERS-active" medium is a citrate-reduced silver colloid. This is because adsorption-induced low- to high-spin-state conversion appears to be minimized, if not eliminated, with this sol, probably because of an organic layer of citrate on the sol surface (Figure 1). Furthermore, antibody-coated silver and gold

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colloids are routinely used in immunology. Indeed, protein A–gold colloid conjugate immunolabeling of anti-P-450 IgG bound to liver microsomes has been used in the determination of the membrane topology of rat liver cytochrome P-450.[16] Therefore, the biocompatibility of these modified metal substrates has been established. However, work by Hildebrandt and Stockburger[17] has demonstrated that conditioning of a bare silver metal electrode surface greatly affects the structure and hence spin state of adsorbed cytochrome *c*. Potential-controlled adsorption is chosen as the means by which the native structure of the protein can be preserved upon binding to the metal surface.[17a] In the same study, the membrane mimetic aspect of protein adsorption on surfaces is indicated. Similar conformational, and hence spin-state, perturbation of cytochrome *c*, as observed with the protein adsorbed on a silver electrode, is revealed upon its interaction with other redox proteins,[17b] large inorganic polyanions, or phospholipid vesicles.[17c] As with the silver electrode study, we have found that exercising careful control over the preparation and size of the silver sol particles and adjusting the conditions (pH, presence of buffer) of the protein/sol interaction can enable the protein to exist in its native form at the metal colloid surface. Similar considerations may apply to the observation of the reversible electrochemistry from cytochrome *c* at modified gold electrodes[18] contrasted with the behavior of irreversibly adsorbed protein on bare gold.[19] Reversible electrochemical behavior is also observed from graphite[20] and metal oxide electrodes.[21] Interestingly, quasireversible, heterogeneous electron-transfer kinetics for cytochrome *c* at smooth and roughened bare silver electrodes have been reported.[22] This indicates that in these cases denaturation does not occur upon protein adsorption and desorption to and from the metal surface.

Unlike the SERRS spectra of the protein in the "native" or "state I" conformation reported in ref 17, we obtain spectra from cytochrome *c* adsorbed on silver sols which differ in the *relative intensity* (but *not* wavenumber) of bands from that obtained in solution.[23] The differences between the solution resonance Raman scattering (RRS) spectra and SERRS spectra from silver colloids have been attributed[23] to the surface selection rules for enhanced Raman scattering.[24] From an analysis of the SERRS band intensities, the heme plane of the absorbed cytochrome *c* is deduced to be parallel to the metal surface. The overall surface charge of the silver tol particle is probably negative due to the presence of strongly adsorbed citrate ions (Figure 1). Independent work using two different techniques[25,26] has suggested that cytochrome *c* may adopt an orientation such that the heme plane is parallel to a *negatively* charged surface. Determination of the orientation of the heme from the application of surface selection rules in analysis of the SERRS intensities may enable the topography of the protein on the sol surface to be deduced.

Experimental Section

The cytochromes P-450 used in this study were purified from liver microsomes from phenobarbital-treated or methylcholanthrene-treated rats as described previously.[33] All the enzymes were of high purity and gave a single band on SDS–polyacrylamide gel electrophoresis.

Silver citrate sols[27] were formed according to the method of Lee and Meisel.[28] Distilled water (500 mL) containing 90 mg of $AgNO_3$ was brought to boiling; 10 mL of freshly prepared 1% sodium citrate was added to the *gently* boiling solution for 1 h. After this time, the reaction was complete, with no change in λ_{max} (approximately 408 nm) for the sol upon further heating. The sols were stable over the course of several months but could also be prepared freshly to ensure consistency and eliminate any effect of aggregation due to aging. If ascorbic acid was used, a *fresh* 1% solution was prepared. Phosphate buffer (0.1 or 0.2 M) was used to subsequently neutralize the sol.

Raman spectra were obtained from sols in a 10-mm fluorometer cell using a 90° scattering geometry and 5-cm^{-1} slits. The samples were not spun as the SERRS signals were stable during the accumulation time (approximately 10 min or longer) of the scanned spectrum. Cytochrome P-450 samples were supplied in 10 mM phosphate buffer containing 20% w/w glycerol and 0.1 mM each of ethylenediaminetetraacetic acid and dithiothreitol. The concentration of protein varied from one cytochrome P-450 sample to the other but was approximately 20×10^{-6} M. In all cases, 10 μL of the cytochrome P-450 sample was added to the sol (3 mL), followed by neutralization (pH 7.0–7.5) with 100 μL of 200 mM phosphate buffer. Acidification was performed *before* and rapid neutralization *after* cytochrome P-450 was added to the sol. Substrate (2 mM benzphetamine) was added to the adsorbed cytochrome P-450 prior to neutralization to yield a 0.1 mM final concentration in the sol. The sol temperature was maintained at 0–5 °C during the course of the Raman measurement by passing cold nitrogen gas over the face of the fluorometer cell.

The 457.9- or 514.5-nm lines of a Spectra Physics 2020 argon ion laser were used as excitation sources at 100 mW. The spectrometer was an Anaspec-modified Cary 81 equipped with a cooled photomultiplier and photon counting detection system.

Results and Discussion

The colloid chosen for this study is a citrate-reduced silver sol. Sodium citrate is used to reduce silver nitrate in water to produce a sol containing silver particles whose average diameter is smaller than the wavelength of visible light (30 nm as determined by photon correlation spectroscopy). Surrounding each particle is a layer of adsorbed citrate, as evidenced by the observation of SERS of citrate ions from a sol that has been aggregated with dilute HNO_3. This layer will stabilize the sol particles by preventing aggregation at too early a stage, since this would ultimately lead to precipitation of the sol. It appears to act as a coating preventing direct protein to silver bonding and enabling interactions with the peripheral amino acid groups of proteins via the hydroxyl and carboxylate groups of the adsorbed citrate ions. With heme proteins, this layer isolates the heme pocket from the metal colloid surface and probably prevents the heme extraction that is observed with borohydride-reduced sols.[29] Further, since this sol helps to maintain protein integrity, it should aid in preserving biological activity. These advantages we broadly classify under the term biological compatibility.

Previous work using borohydride-reduced sols in the presence of excess citrate ions[30] indicated that only a monolayer of citrate ions exists at the silver particle surface. This is probably the case with citrate-reduced sols as well, and similar SERS signals can be obtained.[27a] The signal is not seen at neutral pH because the

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sol is not aggregated, but it can be seen if nitric acid is added to aggregate the sol. As well as acting as a spacer between the protein and the silver metal surface, the layer of citrate ions will influence the electrostatic factors that establish whether an initial attractive colloid/protein interaction occurs. The sol surface in the present case is depicted as a positively charged silver surface coated with negatively charged citrate ions producing a negative effective surface for interaction with the protein. Thus, to obtain a successful protein/sol interaction, a positively charged protein surface is required. Adjusting the pH on either side of the isoelectric point (pI) results in the creation of positive (pH < pI) or negative (pH > pI) charge on the surface.

The peripheral carboxylate groups of the protein are unlikely to be affected by the addition of a few microliters of ascorbic acid. Rather, it is the protonation of NH_2 groups to give NH_3^+ that influences the overall protein surface charge and hence initial bulk electrostatic interactions. The NH_3^+ groups would be expected to interact favorably with the negatively charged citrate ions on the aggregated colloid surface. Therefore, the conditions required for binding of proteins to the sol surface used here will be that pH < pI.

Cytochrome c (pI = 10)[31] at pH 7 possesses sufficient positive charges on its surface to enable it to interact favorably with the sol. Aggregation of the sol particles ensues. This is a necessary condition for strong SERRS and is reflected in color and turbidity changes when the proteins are added to the sols. The stronger signals observed when preacidification is employed may result from the greater number of positive charges on the protein surface, engendering extensive sol aggregation. Due to their lower pI values,[32] phenobarbital-induced microsomal P-450s may not carry sufficient positive charges on their surfaces at pH 7.0 to interact strongly with the sol surface. Aggregation would then not be induced and no detectable SERRS signals generated. Lowering the pH by 0.5–1.0 unit with ascorbic acid enables the protein to favorably interact with the sol surface as more positive charges are created on the integument.

Lowering the pH of the sol may also cause protonation of some of the citrate carboxylate anions (pK_1 = 3.1, pK_2 = 4.8, pK_3 = 6.4) on the sol surface. This would decrease somewhat the negative charge and facilitate sol/protein binding. It is likely that a combination of adjusting the peripheral protein and sol charges is involved in the interactions. However, the effect of changing sol surface charge should be similar for cytochrome c and P-450, and hence the determining factor is likely to be the pI values of individual heme proteins and their constituent outer amino acid residues.

1. SERRS from Two Structurally Related Phenobarbital-Induced Rat Liver Cytochromes P-450. SERRS from cytochromes P-450 IIB1 and IIB2 indicate that the structurally induced spin-state differences between the proteins are preserved at the sol surface.[33] Further analysis of the spectra establishes that differences in bands attributable to peripheral vinyl C–H bending and C_b–S stretching modes are observed between the proteins (where C_b refers to the outer pyrrole carbon of the porphyrin macrocycle and S is a substituent carbon atom). Since control of sol pH upon addition of the protein is a prerequisite to obtain good quality signals from undenatured cytochromes, various methods of altering pH were investigated. Initially the pH of the sol was adjusted by using a small quantity of 1% ascorbic acid followed by rapid neutralization to approximately pH 7.5 using 0.1 M NaOH. Later the method of neutralization was modified by replacing the NaOH with phosphate buffer. A comparison of the SERRS spectra obtained with 514.5-nm excitation from the predominantly low spin cytochrome P-450 IIB1 using NaOH (A) vs phosphate (B) neutralization methods is made in Figure 2. There are differences in the relative intensity and position of

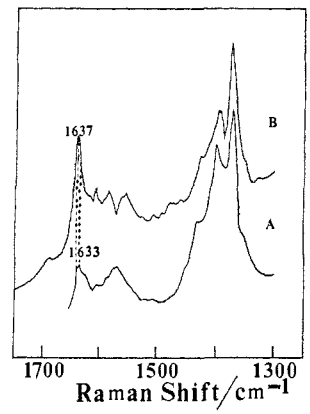

Figure 2. SERRS from 10^{-7} M cytochrome P-450 IIB1 adsorbed on a citrate-reduced silver colloid. The sol has been neutralized to pH 7.5, following preacidification of the colloid with 35 μL of 1% ascorbic acid and (A) 1 drop of 0.1 M NaOH or (B) 100 μL of 200 mM potassium phosphate buffer. λ_{ex} = 514.5 nm; 100-mW power.

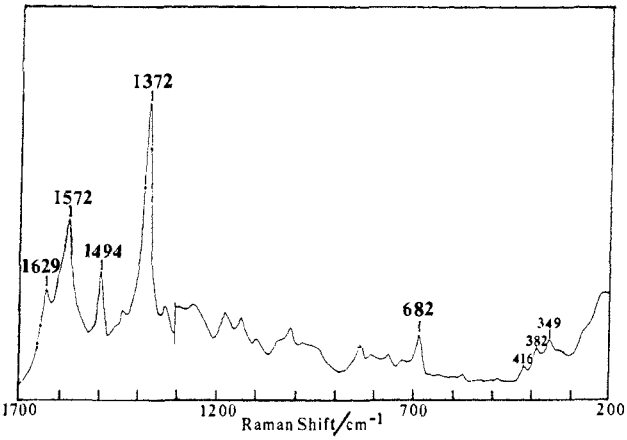

Figure 3. SERRS from 10^{-7} M cytochrome P-450 IIB1 which is adsorbed on a citrate-reduced silver colloid (pH 7.5) after preacidification of the colloid with 65 μL 1% ascorbic acid. λ_{ex} = 457.9 nm; 100-mW power.

the ν_{10} marker band, at approximately 1635 cm^{-1}. With phosphate buffer neutralization, better resolution of the ν_{10} marker band at 1637 cm^{-1} is demonstrated, and an unambiguous assignment of the band to low-spin heme can be made. The C_a–C_m force constant, where a refers to the inner pyrrole carbon of the macrocycle and m to the methine bridging carbon, contributes substantially to the potential energy distribution of ν_{10} and is sensitive to the effect of heme doming or expansion/contraction.[15] After NaOH neutralization, the heme group may lie closer to the sol surface due to a different protein orientation compared to that obtained by neutralization with phosphate buffer. This may create a small degree of protein unfolding, creating microheterogeneaty in the protein and broadening the band due to ν_{10}. Despite this, the method does lead to spin-state conservation.[2k] Phosphate neutralization, however, appears to lead to a different, less strained protein conformation. It is possible that some of the phosphate anions, which are present in 100 times the citrate concentration, occupy a region between the protein and the sol surface, reducing protein unfolding. That the phosphate anions are present on the sol surface is evidenced by the emergence of SERS bands attributable to adsorbed phosphate at 1100 cm^{-1} (broad band) and 720 cm^{-1} [33] (see Figure 5).

Acidification with excess, i.e. >45 μL, 1% ascorbic acid leads to substantial low- to high-spin-state conversion (Figure 3, ν_{10} high spin is expected at 1627 cm^{-1}). More extensive protein unfolding may be occurring, and this may ultimately lead to the biologically inactive form of cytochrome P-450, cytochrome P-420. Evidence that the heme approaches closer to the sol surface with decrease in pH is provided by the increasing overall intensity of the Raman

(31) Ferguson-Miller, S.; Brautigan, D. L.; Margoliash, E. In *The Porphyrins*; Dolphin, D., Ed.; Academic Press: New York, 1979; Vol. 7B, pp 149–240.
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(33) Wolf, C. R.; Seilman, S.; Burke, M. D.; Rospendowski, B. N.; Kelly, K.; Smith, W. E. *Biochemistry* **1988**, *27*, 1597–1603.

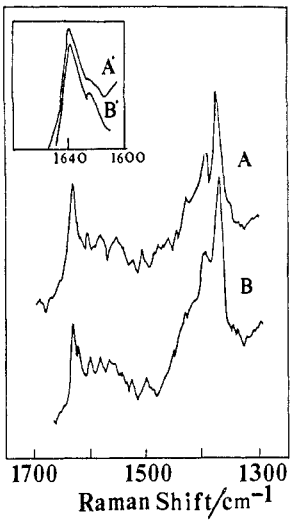

Figure 4. SERRS from 10^{-7} M cytochromes P-450 IIB1 (A) and IIB2 (B) which are adsorbed on citrate-reduced silver colloids (pH 7.5). The inset displays the spectra recorded after expansion of the wavenumber region about ν_{10}. λ_{ex} = 514.5 nm; 100-mW power.

signals as the pH is lowered. Previous studies using nonresonant spacers between a silver surface and a resonant Langmuir–Blodgett film[34] indicated the sensitivity of surface enhancement to distance from the metal surface.

Unfortunately, at this stage we are unable to obtain good quality resonance Raman spectra from the liver microsomal cytochrome P-450 samples available to us. Therefore, a detailed interpretation of the relative intensities of the SERRS spectra, as done with cytochrome *c*,[23] is precluded. However, the overall impression gained from the SERRS spectra from cytochromes P-450 IIB1 and IIB2 excited at 514.5 nm with a final sol pH of 7.5 (Figure 4) suggests a less horizontal orientation with respect to the surface than that of the heme in cytochrome *c*. In particular, this is indicated by the selective enhancement of the $\nu_{10}(B_{1g})$ bands observed with 514.5-nm excitation at 1637 and 1627 cm^{-1}. The ν_{13} and ν_{21} bands are not resolved for these proteins. In the case of cytochrome *c*,[23] selective de-enhancement of these bands was adduced as indicative of a flat orientation of the heme plane. However, solution RRS spectra from phenobarbital-induced rabbit liver cytochromes P-450,[14a] excited at 488 nm, demonstrate that these modes are silent for these proteins. Therefore, no conclusions concerning heme orientation should be made on the basis of these modes.

We propose that the weaker SERRS signals from the cytochromes P-450 as compared to cytochrome *c* are attributable to the more embedded nature of the heme in these proteins. Poulous et al. have published a fully determined X-ray crystallographic structure of bacterial camphor-bound[35] and substrate-free[36] cytochrome $P\text{-}450_{cam}$, from which they deduce the overall topography and environment of the heme group. They conclude that the "closest approach" of the heme to the surface occurs at the proximal face, a distance of about 8 Å. In contrast, the heme group in cytochrome *c* is known to approach the surface of the protein such that one hydrophobic edge is exposed to the solvent.[37] Thus, assuming that the heme is buried deeper in liver microsomal cytochromes P-450, lower SERRS signals are expected. However, maintaining a large distance between the heme and silver surface may be critical in avoiding denaturation and undesirable metal-to-heme charge transfer, and consequently the weaker signals may be a necessary prerequisite to retaining full biological integrity of the less robust P-450s.

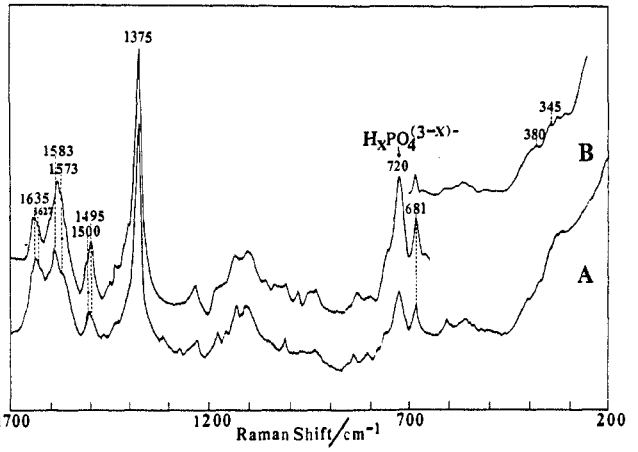

Figure 5. SERRS from 10^{-7} M cytochromes P-450 IIB1 (A) and IIB2 (B) which are adsorbed on citrate-reduced silver colloids (pH 7.5). λ_{ex} = 457.9 nm; 100-mW power.

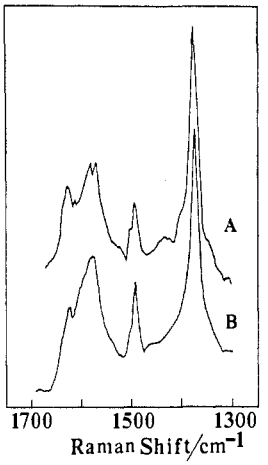

Figure 6. SERRS from 10^{-7} M cytochromes P-450 IIB1 (A) and IIB2 (B) which are adsorbed on citrate-reduced silver colloids (pH 7.5) and which are in the presence of 0.1 mM benzphetamine. λ_{ex} = 457.9 nm; 100-mW power.

Biological activity of the surface-bound protein may readily be determined from SERRS. Addition of the substrate, benzphetamine (0.1 mM final concentration), to either protein adsorbed on the silver sol leads to low to high heme spin-state conversion. Electronic spectra indicate that the mixed low- and high-spin protein, cytochrome P-450 IIB2, in buffered solution is almost completely converted to the high-spin form upon substrate addition.[33] The predominantly low spin cytochrome P-450 IIB1 undergoes only partial conversion in the presence of benzphetamine. This behavior is reflected in the SERRS experiment for both isozymes.

The ν_3 spin-state maker band, resolved most clearly with 457.9-nm excitation, shows the effect of substrate on the spin-state equilibrium. Native cytochrome P-450 IIB1 (Figure 5A) gives rise to essentially a single band at 1500 cm^{-1}, while that found with the isozyme IIB2 (Figure 5B) has a peak at 1495 cm^{-1} with a shoulder at 1500 cm^{-1}. Substrate addition modifies the band such that a peak at 1494 cm^{-1} with a medium-intensity shoulder at 1500 cm^{-1} emerges for IIB1 (Figure 6A). Substrate addition to IIB2 (Figure 6B) shows a peak at 1494 cm^{-1} with only a weak shoulder at 1500 cm^{-1}. Other spin-state marker bands differentiate between the effects of adding substrate to the two isozymes. For IIB1 there are two peaks in the region where the ν_2 band would be expected, at 1583 and 1574 cm^{-1}. This indicates the conservation of substantial low-spin heme in the presence of substrate. The ν_2 band for IIB2 is not resolved into two distinct bands, indicating greater low- to high-spin-state conversion upon substrate

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(35) (a) Poulos, T. L.; Finzel, B. C.; Gunsalus, I. C.; Wagner, G. C.; Kraut, J. *J. Biol. Chem.* **1985**, *260*, 16122–16130. (b) Poulos, T. L.; Finzel, B. C.; Howard, A. *J. Mol. Biol.* **1987**, *195*, 687–706.
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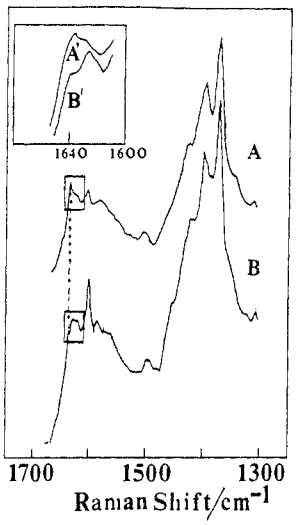

Figure 7. SERRS from 10^{-7} M cytochromes P-450 IIB1 (A) and IIB2 (B) adsorbed on citrate-reduced silver colloids (pH 7.5) following addition of 0.1 mM benzphetamine. The insets display the spectra recorded after expansion of the wavenumber region about ν_{10}. λ_{ex} = 514.5 nm; 100-mW power.

addition. As with the native proteins, the ν_{10} band is probably obscured with 457-nm excitation by overlap with the C=C(str) band of peripheral vinyl groups, and the ν_2 band is broadened further by overlap with the ν_{19} band the possible SERRS activation of the ν_{38} band.

Unambiguous assignment of the band at 1637 cm^{-1} to ν_{10} low spin is facilitated by 514.5-nm excitation (Figure 4A,B). With IIB2, the shoulder at 1627 cm^{-1} (ν_{10} high spin) is in keeping with the greater high-spin content of this protein. For IIB1, ν_{10} gives rise to a single peak, with little indication of peaks due to high-spin protein. Expanding the 1600–1650-cm^{-1} region (see insets, Figure 4A′,B′) enables the high-spin component to be better discerned. Upon substrate addition, the spectrum in this region alters. IIB1 (Figure 7A,A′) undergoes partial low- to high-spin-state conversion, reflected in the emergence of a broad ν_{10} band, composed of both low and high spin marker bands. IIB2 (Figure 7B,B′) displays more complete conversion to the high-spin form with a decrease of the 1637-cm^{-1} band and emergence of a peak at 1627 cm^{-1}. A secondary feature at 1605 cm^{-1}, particularly prominent with IIB2, is attributable to benzphetamine itself. Whether it is due to protein-bound benzphetamine or directly adsorbed substrate on the silver sol remains to be determined. However, the peak is in the same position as is found in the SERS of adsorbed benzphetamine (not shown) in the absence of protein, and the large excess of substrate should enable it to compete effectively for adsorption sites on the silver colloid. Thus, the evidence is weighted in favor of silver- rather than protein-bound substrate.

Thus, the observation from UV–vis measurements that cytochrome P-450 IIB2 undergoes much more substantial substrate-induced low- to high-spin-state conversion than IIB1[33] is reflected in the SERRS experiment. However, more quantitative comparisons are difficult because of the enhancement mechanisms which obtain in the resonance contribution to the intensity amplification. There is no simple correlation between relative signal intensities and relative amounts of low- and high-spin-state protein. The absolute Raman cross section depends on the polarizability of the molecule, which in turn is related to Franck–Condon and/or Herzberg–Teller overlap integrals between different vibronic states in the molecule. At resonance, particular normal coordinates can assume greater significance than others, enabling better overlap between the ground and excited states. Low- to high-spin-state conversion is accompanied by alteration in the heme geometry as well as by a hypsochromic shift in λ_{max}. Thus, scattering from normal modes may be preferentially enhanced upon the transition from low- to high-spin heme. Scattering from the normal mode $\nu_3(A_{1g})$ is selectively enhanced for high-spin ferric heme with 457.9-nm excitation, whereas that from ν_{10} appears to be preferentially enhanced with low-spin heme excited at 514.5 nm. This may be a consequence of greater Franck–Condon overlap integrals for the high-spin state, enhancing scattering from the totally symmetric ν_3 mode with Soret band, 457.9-nm excitation. This effect could be larger than any low- to high-spin-state-induced hypsochromic shift of the B-band λ_{max}. The greater oscillator strength of the B-band of high-spin heme compared to low-spin heme may enable an increased contribution of Franck–Condon scattering for the former with 457.9-nm excitation. However, other high-spin marker bands due to totally symmetric modes, e.g., the ν_2 band, would be expected to be selectively enhanced if this explanation held. Thus, this rationalization does not fully account for the specificity of the enhancement of the ν_3 band. More detailed consideration of the electronic ground- and excited-state orbital geometries and specific vibrational displacements may be required to explain these effects fully.

Selective enhancement of the low-spin-state marker band, ν_{10}, with 514.5-nm excitation cannot be explained on the basis of the oscillator strength of the electronic transition in this region. The high-spin-state form created by substrate addition to IIB2 has the same or greater extinction coefficient at this wavelength due to the α-band of the Q-band system. Possibly, the hypsochromic shift in ν_{max} for the Soret band of the high-spin heme leads to less effective Herzberg–Teller coupling of the B- and Q-bands. Scattering from non totally symmetric modes gains intensity from such coupling. The UV–vis spectrum in the Q-band region does indicate that the intensity of the α-band is lowered for the high-spin protein. This is consistent with less effective coupling of the Q- to the B-band, from which the former borrows intensity. However, the β-band of the Q-band has a slightly raised intensity. (The β-band is the vibronically active side band in the Q-band region.) Using 514.5-nm excitation, which is close to resonance with the Q-state 0-1 vibronic transition for ν_{10} (0 and 1 refer to the ground electronic state with vibrational quantum number = 0 and excited electronic Q-state with vibrational quantum number = 1, respectively), should yield strong resonance Raman scattering intensity for ν_{10}. As the intensity of this vibronic band does not decrease when the low-spin heme is converted to the high-spin state, the resonance Raman scattering intensity of the ν_{10} band (which gains its intensity from the same vibronic origin) should not decrease. Thus, the ν_{10}-band intensity may be a more representative measure of the relative amounts of low- and high-spin heme, as it appears to be equally enhanced for both spin states.

2. SERRS from Other Cytochromes P-450. The two structurally related cytochromes P-450 from phenobarbital-treated rat liver studied in section 1 have low and mixed high- and low-spin states, respectively. Methylcholanthrene-induced rat liver microsomal cytochrome P-450, P-450 IA2, is predominantly a high-spin protein. It was adsorbed onto a silver colloid and the SERRS spectrum recorded. As with the phenobarbital-induced cytochromes P-450, acidification of the sol preceded addition of the protein. Marked improvement of the spectral quality accompanied use of greater than 35 μL of 1% ascorbic acid, especially at 457.9 nm. As discussed earlier, irreversible cytochrome P-450 to P-420 conversion is responsible for the increased SERRS. Consequently, less acid (17.5 μL) was used to maximize the SERRS signal without protein denaturation.

IA2 (10 μL of 20 μM protein) was adsorbed on the silver citrate sol (3 mL), preacidified with the minimum necessary acid (17.5 μL of 1% ascorbic acid) and neutralized with buffer to yield the SERRS spectrum shown in Figure 8A. A number of peaks are assigned to spin-state marker bands characteristic of five coordinate high-spin heme (for example, ν_3–1494 cm^{-1}; ν_2–1578 cm^{-1}, and ν_{10}–1629 cm^{-1}). A small amount of low-spin protein may be present as indicated by slight broadening of the ν_3 band. The ν_2 band is probably broadened by the breakthrough of the ν_{19} band due to surface depolarization and possible SERRS activation of ν_{38}. Overlap of the ν_{10} band with the vinyl C=C(str) band is probably responsible for the broadening at about 1629 cm^{-1}. The spectrum is of poorer quality than that from the phenobarbital-

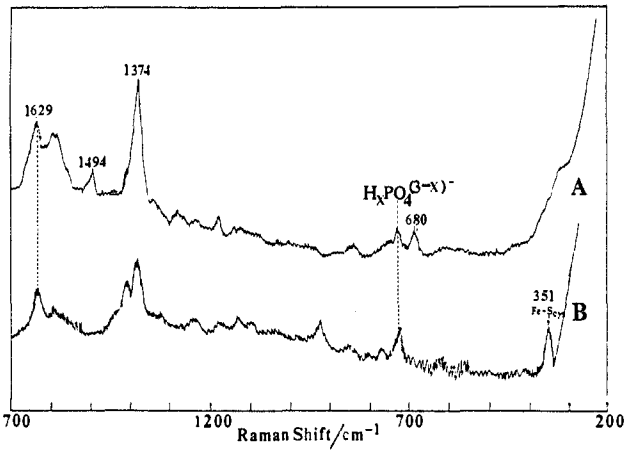

Figure 8. SERRS of 10^{-7} M cytochrome P-450 IA2 which is adsorbed on a citrate-reduced silver colloid (pH 7.5) and which is excited at (A) 457.9 and (B) 514.5 nm; 100-mW power.

induced cytochromes P-450, possibly due to a blue shift of the Soret band maximum. Below 1300 cm^{-1} only a weak band at 680 cm^{-1}, assigned to $\nu_7(A_{1g})$, can be discerned.

Excitation at 514.5 nm produces a poorer signal-to-noise ratio (Figure 8B) than 457.9-nm excitation. A very large background is manifested. However, the major spin- and oxidation-state markers are clearly resolved (1629 cm^{-1}–ν_{10} and 1374 cm^{-1}–ν_4). Weaker bands at 1400 cm^{-1} [$\nu_{20}/\nu_{29}(A_{2g}/B_{2g})$] and 1174 cm^{-1} [$\nu_{30}(B_{2g})$] may be discerned.

A strong band is resolved in the low-wavenumber region at 351 cm^{-1} with 514-nm excitation. Camphor-bound cytochrome P-450_{cam} has been shown to behave similarly in solution resonance Raman studies.[38] As with the liver microsomal IA2, a strongly enhanced band at 351 cm^{-1} is found with 514.5-nm excitation of P-450_{cam}. This band is assigned as a Fe–S_{cys} stretching mode.[38] An electronic charge-transfer band underlying the Q-band region is believed to be responsible for the singular resonance enhancement of this low-wavenumber Raman band. Charge transfer to the Fe(III) d_{z^2} orbital from the sulfur d_{z^2} orbital is proposed. Champion et al.[39] report a strong Raman band at 351 cm^{-1} for camphor-bound P-450_{cam} using 363-nm excitation. They assign this band to Fe–S_{cys}(str).[39b] This is confirmed from ^{56}Fe–^{54}Fe and ^{32}S–^{34}S isotopic substitution experiments. Unfortunately, the authors do not report the isotopic substitution effect on the 351-cm^{-1} band enhanced with 514.5 nm. However, the strong likelihood is that the 351-cm^{-1} band observed with 514.5-nm excitation may be assigned to Fe–S_{cys}(str). The observation of two Z-polarized bands in the UV–vis spectrum of camphor-bound P-450 at 323 and 567 nm[40] lends support to this argument. It has been suggested that charge-transfer transitions are responsible for these bands. Assuming the thiolate iron band stretching assignment is correct, compelling evidence for undenatured protein has been uncovered. Thiolate–iron bond cleavage would probably be the first step in any denaturation pathway upon adsorption onto the sol. Thus, the presence of scattering from the Fe–S_{cys}(str) in the SERRS spectrum indicates that cytochrome P-420 production or heme extraction has been avoided.

An alternative assignment of the 351-cm^{-1} band would be to ν_8, a mode described as due to mainly C_b–S in-plane bending. This would appear to be the case with cytochrome *c*. With 514.5-nm excitation, the strongest low-wavenumber band, at 355 cm^{-1}, is attributable to ν_8. However, a rich spectrum in the low-wavenumber region is obtained from cytochrome *c* with resonance Raman scattering.[41] Several relatively intense bands appear

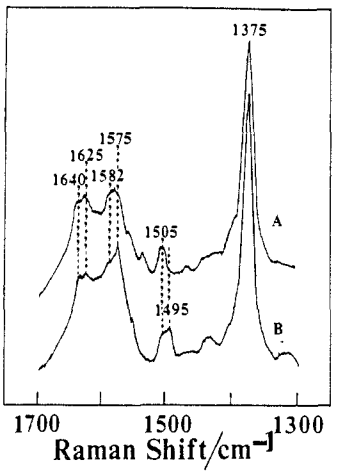

Figure 9. SERRS from 10^{-7} M cytochrome P-450 IIB4 which is adsorbed on a citrate-reduced silver colloid (pH 7.5) and which is in the absence (A) and presence (B) of 0.1 mM benzphetamine. λ_{ex} = 457.9 nm; 100-mW power.

alongside ν_8 in the 300–500-cm^{-1} region. Cytochrome P-450 IA2 yields only a single band in the 300–600-cm^{-1} region. The uniqueness of the band seems to favor the previous explanation of a selective enhancement mechanism arising from thiolate S to Fe(III) high-spin charge transfer.

Another cytochrome P-450, IIB4, was studied by using SERRS. The protein is extracted from rabbit liver and is phenobarbital inducible. It is known to exist predominantly in the low-spin state in aqueous glycerol buffer. Recently, work published by Hildebrandt et al.[42] indicated that substantial low- to high-spin-state conversion occurred when the protein was reconstituted in phospholipid vesicles and adsorbed on silver sols. When the benzphetamine-bound reconstituted protein was adsorbed at the sol surface, spectral changes consistent with substrate-induced low- to high-spin-state conversion were observed. However, substantial low- to high-spin-state conversion had already been engendered by adsorption to the silver surface. Consequently, the change was detected mainly as a reduction in intensity of the low-spin-state ν_3 marker band on the shoulder of the lower wavenumber sol-induced high-spin heme peak.

By use of the sol prepared in this laboratory, the SERRS experiment with cytochrome P-450 IIB4 was repeated, with the exception that reconstituted protein was replaced by "native" glycerol solubilized material. Since it would be expected that reconstitution would confer a degree of protection from the sol-induced low- to high-spin-state conversion, especially if, as suggested by the authors, electrostatic interactions at the adsorbate/silver interface are responsible for the spin-state change, it was possible that the native protein would be almost completely converted to the high-spin form. However, SERRS from essentially intact protein, with very little or no low- to high-spin-state conversion, was obtained (Figure 9A).

When the preacidification procedure was adopted to "activate" the sol before protein addition, the signal-to-noise ratio was improved by up to 3 times without any changes in relative intensity or band position. Unlike the rat liver cytochromes P-450, Raman signals for this protein could be detected without any acid treatment. Preacidification in excess of the amount required to obtain the optimum signal resulted in substantial low- to high-spin-state conversion and ultimately cytochrome P-420 production for the cytochrome P-450 IIB4 protein. Less acid than that required to reach this optimum with the rat liver cytochromes P-450 was required. This may indicate greater lability of the thiolate–iron bond in rabbit liver P-450. Along with the observation that SERRS signals could be detected in the absence of

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Table I. Main Features of the SERRS Spectra of Four P-450s Including the Spin- (ν_{10}) and Oxidation-State (ν_4) Marker Bands

cyclochrome-P-450	high wavenumber SERRS band position,[a] cm^{-1}, and mode assignment (symmetry type)					
	ν_{10} (B_{1g})	C=C(str)	ν_2 (A_{1g})	ν_3 (A_{1g})	ν_{20} (B_{2g})/ν_{29} (B_{2g})	ν_4 (A_{1g})
IIB1	1637 ls 1627 hs[sh]	1625	1583 ls 1573 ls[sh]	1500 ls	1399	1374
IIb2 + benz[b]	1637 ls 1627 hs[sh]	1625	1583 ls 1574 hs	1500 ls[sh] 1494 hs	1399	1374
IIB2	1637 ls 1627 hs[sh]	1625	1583 ls 1573 hs	1500 ls[sh] 1495 hs	1399	1375
IIB2 + benz	1637 ls[sh] 1627 hs	1625	1583 ls 1574 hs	1500 ls[wsh] 1494 hs	1399	1375
IA2	1629 hs	1625	1578 hs	1494 hs	1400	1374
IIB4	1640 ls	1625	1582 ls	1505 ls	1400	1375
IIB4 + benz	1637 ls 1629 hs	1625	1582 ls[sh] 1575 hs	1505 ls[sh] 1495 hs	1400	1375

[a] ls = low spin; hs = high spin; sh = shoulder; wsh = weak shoulder. [b] benz = benzphetamine as substrate.

organic acid, this demonstrates that each individual protein must be considered separately and that the SERRS technique must be developed differently to accommodate different biological species. Possibly the isoelectric point of rabbit liver cytochrome P-450 differs from that of its rat liver congeners, and thus the surface is already rendered favorable for adsorption onto the silver surface.

The spectrum in the high-wavenumber region of cytochrome P-450 IIB4 (10 μL of 20 μM) adsorbed on a silver colloid (3 mL) preacidified with 3.5 μL of 1% ascorbic acid and excited with 457.9-nm excitation is displayed in Figure 9A. Neutralization was accomplished, as before, with 100 μL of 200 mM phosphate buffer (pH 7.5). Below is the spectrum obtained by the addition of benzphetamine (0.1 mM final concentration) using the same excitation wavelength (Figure 9B). Native, substrate-free cytochrome P-450 IIB4 (Figure 9A) displays all the bands characteristic of low-spin, six-coordinate heme. The ν_3 band appears at 1505 cm^{-1} and the ν_2 band, broadened due to overlap with the ν_{19} and possibly SERRS-activated ν_{38} bands, at 1582 cm^{-1}. Resolution of the ν_{10} band at 1640 cm^{-1} is prevented by the presence of the band due to C=C(str) of peripheral vinyl groups at approximately 1625 cm^{-1}. Substrate addition produces spectral modifications ascribable to low- to high-spin-state conversion (Figure 9B). The ν_3 band broadens further than in the substrate-free spectrum, peaking at the high-spin marker region at approximately 1495 cm^{-1}. The ν_2 band peaks at 1575 cm^{-1} and is consonant with substrate-induced low- to high-spin-state conversion of the heme group. Above 1600 cm^{-1} resolution is poor and definitive assignments are not possible.

As with the previous proteins studied, using 514-nm excitation to switch the excitation wavelength into, or just off, resonance with the β-band of the Q-band region increases resolution of ν_{10}. Although there is a considerable background, expansion of the Raman spectra about the ν_{10} frequency reveals a single symmetric band around 1640 cm^{-1} for the native, substrate-free protein (see Figure 10A′, inset). Displayed below this spectrum is that obtained from the adsorbed protein after substrate addition (Figure 10B′, inset). This inset enables direct comparison of the expanded ν_{10}-band region for both species. Substrate-bound cytochrome P-450 IIB4 exhibits a low-spin marker ν_{10} band at 1637 cm^{-1} with an auxillary feature at 1629 cm^{-1}. This latter band is assigned as ν_{10} high spin, indicating that low- to high-spin-state conversion has accompanied benzphetamine addition. As before, the extent of spin-state conversion suggested by ν_{10} observed with 514.5-nm excitation (Figure 10) is considerably lower than that indicated by the ν_3 band with 457.9-nm excitation (Figure 9). According to the argument made earlier, that ν_{10} band, enhanced at 514.5-nm excitation, is a more quantitative measure of spin-state conversion, approximately 30% of the adsorbed low-spin protein is converted to the high-spin form in the presence of 0.1 mM benzphetamine.

A significant shift in wavenumber of ν_{10} low spin is detected in the SERRS spectrum when benzphetamine is bound. Substrate-free protein exhibits ν_{10} at 1640 cm^{-1}, whereas ν_{10} appears

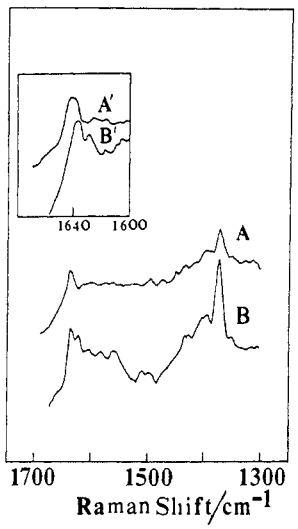

Figure 10. SERRS from cytochrome P-450 IIB4 adsorbed on a citrate-reduced silver colloid (pH 7.5) in the absence (A) and presence (B) of 0.1 mM benzphetamine. The inset displays the spectra recorded after expansion of the wavenumber region about ν_{10}. λ_{ex} = 514.5 nm; 100-mW power.

at 1637 cm^{-1} for substrate-bound protein.[43] The 3-cm^{-1} shift may be the result of a different low-spin species existing in equilibrium with the high-spin cytochrome P-450 IIB4 benzphetamine complex. Possibly the benzphetamine is still present at the active site, even in the low-spin form. This would require some conformational change about the heme site. This may involve displacement of water molecules, which have been shown in the case of $P\text{-}450_{cam}$[35] to occupy the vacant active site in substrate-free protein. The hydroxy group, which is thought to ocupy the sixth coordination site on the heme, must remain to preserve the six-coordinate low-spin state. However, the hydrogen-bonded network of water molecules would be disrupted by the presence of benzphetamine, and this may be indicated by the 3-cm^{-1} downward shift in ν_{10}.

Conclusion

SERRS from biologically active cytochromes P-450 can be obtained if the correct conditions are established before and after introduction of the proteins to the silver colloidal medium. To

(43) During the course of the review process of this paper, two resonance Raman scattering studies have appeared. First, the comparative active structures of substrate band and free cytochromes P-450 LM2 and LM4 and, second, the effect of substrate binding to cytochromes P-450 LM2 of monomeric as compared to oligomeric protein forms have appeared in: (a) Hildebrandt, P.; Greinert, R.; Stier, A.; Taniguchi, H. *Eur. J. Biochem.* **1989**, *186*, 291–302. (b) Hildebrandt, P.; Garda, H.; Stier, A.; Bachmanoea, G. I.; Kanaeba, I. P.; Archakob, A. I. *Eur. J. Biochem.* **1989**, *186*, 383–388.

achieve this, it is necessary to carefully control the temperature and nature of the colloid surface coating. This latter condition is well met by using citrate-reduced sols. Adsorbed citrate ions coat the silver surface and confer a protein-compatible surface. This organic layer may prevent the heme approaching too closely to the silver surface, especially when a pH < pI is used to activate a protein/colloid interaction. This is critical in avoiding denaturation by heme extraction, in cytochrome P-420 formation, or in avoiding undesirable low- to high-spin-state conversion. The main features of the spectra of each of four P-450s studied are summarized in Table I. That the cytochromes P-450 are relatively intact and biologically active on the sol surface is exemplified by the response to substrate of the adsorbed proteins.

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Registry No. Ag, 7440-22-4; cytochrome P450, 9035-51-2; benzphetamine, 156-08-1; citric acid, 77-92-9; heme, 14875-96-8.

Intrinsic Barriers to Atom Transfer: Self-Exchange Reactions of $CpM(CO)_3X/CpM(CO)_3^-$ Halide Couples

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Abstract: Rate constants and activation parameters were measured for the self-exchange of $CpM(CO)_3^-$ with $CpM(CO)_3X$ in CD_3CN solvent:

$$CpM(CO)_3^- + CpM(CO)_3X \rightleftharpoons CpM(CO)_3X + CpM(CO)_3^-$$

The self-exchange reactions were followed by ^1H NMR spectroscopy: For the X = I complexes, standard line width measurements yield (M = Mo) $k(298) = 1.5 \times 10^4$ M^{-1} s^{-1} ($\Delta H^\ddagger = 6.4$ (±0.4) kcal mol^{-1}, $\Delta S^\ddagger = -18$ (±1.5) cal K^{-1} mol^{-1}) and (M = W) $k(298) = 4.5 \times 10^3$ M^{-1} s^{-1} ($\Delta H^\ddagger = 7.5$ (±0.1) kcal mol^{-1}, $\Delta S^\ddagger = -16.8$ (±0.5) cal K^{-1} mol^{-1}). For the X = Br complexes, magnetization-transfer experiments yield (M = Mo) $k(298) = 1.6 \times 10^1$ M^{-1} s^{-1} ($\Delta H^\ddagger = 12.1$ (±4.5) kcal mol^{-1}, $\Delta S^\ddagger = -12$ (±15) cal K^{-1} mol^{-1}) and (M = W) $k(298) = 1.5$ M^{-1} s^{-1} ($\Delta H^\ddagger = 15.1$ (±5.2) kcal mol^{-1}, $\Delta S^\ddagger = -7$ (±16) cal K^{-1} mol^{-1}); ^1H NMR longitudinal relaxation times T_1 for the Cp groups of the reactants are typically 40 s. The X = Cl systems were studied by conventional techniques, with the rates of "transfer" of Cp-d_5 from $(Cp\text{-}d_5)W(CO)_3^-$ to $CpW(CO)_3Cl$ being monitored; for M = Mo, $k(298) = 9.0 \times 10^{-2}$ M^{-1} s^{-1} ($\Delta H^\ddagger = 18.9$ (±1.0) kcal mol^{-1}, $\Delta S^\ddagger = 0$ (±4) cal K^{-1} mol^{-1}) and for M = W, $k(298) = 2.1 \times 10^{-3}$ M^{-1} s^{-1} ($\Delta H^\ddagger = 17.7$ (±3.3) kcal mol^{-1}, $\Delta S^\ddagger = -11$ (±11) cal K^{-1} mol^{-1}). For X = CH_3, the $CpW(CO)_3^-/CpW(CO)_3CH_3$ self-exchange rate constant, also determined by monitoring rates of "transfer" of Cp-d_5 from $(Cp\text{-}d_5)W(CO)_3^-$ to $CpW(CO)_3X$, is $\approx 1 \times 10^{-5}$ M^{-1} s^{-1} at 335 K. The latter self-exchange reaction is discussed in terms of the intrinsic barrier for oxidative addition to the anion. For the X = halogen exchanges, the role of the M(I) ("metal radical") state is considered, and it is concluded that the latter isovalent state is not an intermediate for these systems. However, the isovalent state may serve to stabilize the transition state for two-electron transfer between the metal centers through configuration interaction. Our results, taken with those for other X = halogen systems, indicate that effective transfer of X^+ may be intrinsically rapid when both reactants are 18-electron species and steric factors are favorable.

Introduction

The concept of factoring the kinetic barrier to a reaction into intrinsic and thermodynamic components has proven remarkably powerful for outer-sphere electron-transfer reactions.[1] In recent years, this approach has also been applied to proton-transfer, methyl (CH_3^+)-transfer, and other "atom"-transfer reactions.[2,3] In these applicatons, a crucial parameter is the free energy barrier to the self-exchange ($\Delta G^\circ = 0$) reaction, $\Delta G^\ddagger_{ex}$. While $\Delta G^\ddagger_{ex}$ values have been inferred from kinetic data for net ($\Delta G^\circ \neq 0$) reactions, only rarely (with the exception of outer-sphere electron-transfer reactions) have the intrinsic barriers been evaluated by direct study of the self-exchange process. It is becoming apparent that transition-metal complexes are excellent substrates for such studies. In this paper we describe our studies of "atom-transfer" self-exchange reactions of $CpM(CO)_3^-/CpM(CO)_3X$ couples. In our experiments a range of ^1H NMR techniques provide a probe of the self-exchange process analogous to that of radioisotopes in the earliest self-exchange measurements.

Following Taube[4] we adopt the term "atom transfer" for reactions in which an atom originating in either the oxidizing or reducing agent is transferred to the reaction partner so that in the activated complex both oxidizing and reducing centers are attached to the atom being transferred. In this sense, "atom transfer" is intended as a broad reaction class and is not restricted to reactions in which a single neutral atom is transferred (for example, a hydrogen atom or halogen atom abstraction). Reactions falling within this class include one-electron and two-electron inner-sphere electron-transfer reactions,[5] halogen- and hydrogen-atom abstractions, hydride-transfer reactions, and certain proton-transfer and nucleophilic substitution reactions. With, perhaps, one[6] exception, all known two-electron-transfer reactions

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