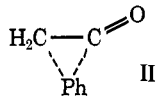
similar g tensors and coupling constants to the α protons. For example, the g values of 2.0044 and 2.0048 and the A_{iso} to the α protons of 19.8 and 18.1 G have been reported for CH_3–CO–CH_2[13] and 2-cyclohexanonyl radicals,[14] respectively. The g_{iso} and A_{iso} computed from Table I are 2.0046 and 19.8 G and are very close to the values cited above. The contribution of the resonance structure of the type Ib must be very similar in these radicals. Based upon the ^{13}C and ^{17}O hyperfine coupling constants, it has been reported[14] that the contribution of the type Ib structure in 2-cyclohexanonyl is about 15%.

The decomposition of I to benzyl upon thermal activation is probably a process unique to the phenacyl radical motivated by the much more favorable resonance stabilization of the benzyl radical. It must go through the cyclic transition state shown below, followed by the decarbonylation. It is not an unexpected transition state since 1–2 migration of an aryl group is one of the most often observed radical rearrangements.



II

Indeed, if structure II with its spin density at the ortho and para positions is the thermodynamically favored one for phenacyl at 125° and if the decarbonylation occurs only at much higher temperature, all of the anomalous results obtained during the attempts to synthesize dibenzoylethane or any of its 2 and/or 3 methyl substituted homologs could be explained. Kochi, *et al.*,[15] have shown that several aliphatic free radicals rearrange at *ca.* −120° in a manner similar to that which is here implied.

Direct evidence for the structure (II) at 125° or possibly at room temperature should be obtainable from esr studies using ultraviolet photolysis of phenacyl iodide or bisazophenacyl in solution *via* flow technique. These investigations are now in progress at one of our laboratories.

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Resonance Raman Scattering from Iron(III)- and Copper(II)-Transferrin and an Iron(III) Model Compound. A Spectroscopic Interpretation of the Transferrin Binding Site[1a]

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Abstract: Laser excitation within the visible absorption band of both the Fe(III) and Cu(II) complexes of human serum transferrin reveals four resonance enhanced Raman bands at 1604, 1505, 1284, and 1173 cm^{-1}. These are assigned to phenolate vibrational modes by comparison with the Fe(III) complex of ethylenediamine di(*o*-hydroxyphenylacetate), EDDHA. The lack of observable frequency shifts upon ^{18}O substitution in bicarbonate confirms that bicarbonate does not contribute significantly to the resonance Raman spectrum. This observation does not preclude direct binding of bicarbonate to iron, but the primary role of bicarbonate in the formation of Fe(III)–transferrin is apparently to induce a protein conformation change which brings the binding groups into proximity. The visible absorption band is assigned to phenolate → Fe(III), $p\pi$–$d\pi^*$ charge transfer (probably $p\pi$–$d\sigma^*$ for Cu(II)), and the close similarity of this band for Fe(EDDHA)$^-$ and Fe(III)–transferrin implies participation of *two* tyrosines in the binding site of the protein. The Raman excitation profiles of Fe(EDDHA)$^-$ and Fe(III)–transferrin show unprecedentedly complex behavior, within which a progression of peaks with a uniform 1000 cm^{-1} spacing can be observed. This is interpreted as reflecting enhanced Raman scattering from successive excited state vibrational levels involving the phenolate C–O stretching mode.

The two binding sites of the serum iron-transport protein transferrin display an extraordinary avidity for iron. With an apparent binding constant, $K = 10^{24}$,[2] transferrin effectively protects its charge of bound iron from hydrolysis and chelation by serum components. Yet upon encounter with a reticulocyte, the binding affinity changes dramatically, and iron is released to a membrane-bound iron receptor site for eventual incorporation into hemoglobin.[3]

Although the detailed physical structure of the iron binding site of transferrin remains largely unresolved, several facts regarding the nature of the site are known. Chemical modifications[4,5] have suggested that tyrosine and histidine participate in metal binding. Spectro-

(1) (a) This work was supported by a grant from Research Corp. and a faculty research fellowship from the University of Michigan (to B.P.G.) and NIH Grant GM13498 and NSF Grant GP41008X (to T.G.S.); (b) the University of Michigan; (c) Princeton University.

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scopic techniques corroborate these observations. Ultraviolet difference spectra[6,7] fluorescence[8] and nmr spectra[9] suggest the perturbation of 2–3 tyrosines per bound metal. Involvement of nitrogen in the formation of the Cu(II) complex of transferrin has been inferred from electron paramagnetic resonance.[10] Measurements of solvent proton spin–lattice relaxation rates demonstrate that a labile water molecule resides on each metal ion in Cu(II)–transferrin.[11]

The binding site is sufficiently flexible to accommodate a variety of metal ions other than ferric iron; these include Cu(II), Cr(III), Mn(III), and Co(III).[12] Ferrous iron, if bound at all, is bound very weakly.[13,14] A remarkable aspect of transferrin chemistry is the binding of one equivalent of bicarbonate[15] per bound metal ion at an anion binding site.[16] Iron(III) does not appear to be bound in the absence of bicarbonate.[17]

The intense visible absorption of many metal–transferrin complexes[12] suggested the application of resonance Raman scattering[18] as a technique for further elucidation of the structure of the transferrin metal-binding site. Two recently published studies[19,20] show that resonance enhanced Raman spectra are indeed obtained upon excitation in the transferrin visible absorption band. Here we report Ruman spectra and excitation profiles for Fe(III)- and Cu(II)–transferrin and of a small-molecule Fe(III) complex which shows strikingly similar spectral characteristics to those of Fe(III)–transferrin.

Experimental Section

Human serum transferrin was prepared and characterized by the methods of Aisen, *et al.*[21] Fe(III)–transferrin (1.48×10^{-4} *M*) and Cu(II)–transferrin (1.73×10^{-4} *M*) solutions were prepared in 0.12 *M* Tris buffer, pH 8, and contained $NaClO_4$ (0.05 *M*) as a frequency and intensity standard.

Oxygen-18 enriched bicarbonate was prepared by addition of solid $NaHCO_3$ to $H_2{}^{18}O$ (83 g atom %, Bio-Rad Lab.); the $NaHCO_3$ concentration was 0.15 *M*. After one week's equilibration[22] at 27°, lyophillized Fe(III)–transferrin (sufficient for 1.5×10^{-4} *M*), solid Tris buffer, and $NaClO_4$ were dissolved in the bicarbonate solution. The protein solution was equilibrated for 1 week[23] at 25°. The control (in $H_2{}^{16}O$) was prepared in parallel with the enrichment experiment. The ^{18}O content of the samples was about 70%, as determined directly from the Raman bands of free bicarbonate in both the protein samples and blanks prepared without Fe(III)–transferrin. Deuterated Fe(III)– and Cu(II)–transferrin were prepared by dissolving lyophillized protein in a solution of D_2O, tris buffer, and $NaClO_4$.

Ethylenediamine di(*o*-hydroxyphenylacetic acid), H_4(EDDHA), obtained from K & K Lab., was purified by repeated cycles of dissolution in concentrated NH_4OH under nitrogen, cautious neutralization with H_2SO_4, filtration of the precipitate, and thorough washing with water. The Fe(III) complex salts were prepared by stirring equimolar quantities of H_4(EDDHA), freshly precipitated hydrous ferric oxide, and the bicarbonate of the desired cation in 3:1 H_2O:methanol under N_2 for about 12 hr. The solutions were filtered, stripped on a rotary evaporator, and the deep brick-red residues recrystallized, first from methanol-ether and then from water. The product crystallizes as bundles of glistening red-black needles or plates. Elemental analyses were performed by Schwartzkopf Microanalytical Lab.

Anal. Calcd for $Na[Fe(EDDHA)]\cdot 4H_2O$: C, 42.45; H, 5.15; N, 5.51. Found: C, 42.41; H, 5.09; N, 5.33. Calcd for $K[Fe(EDDHA)]\cdot 1.5H_2O$: C, 45.01; H, 4.41; N, 5.83. Found: C, 45.57; H, 4.51; N, 5.83. Calcd for $NH_4[Fe(EDDHA)]\cdot 2H_2O$: C, 46.64; H, 5.60; N, 8.97. Found: C, 46.35; H, 5.40; N, 8.83. Infrared spectra (Nujol mulls) of all of the salts support the presence of water of crystallization, with relative intensities of the H_2O bands being roughly in keeping with the above formulas. Solutions for Raman measurements were $1–2 \times 10^{-3}$ *M* and for most runs contained Na_2SO_4 (0.5 *M*) as a standard.

The Raman spectrometer consisted of a Spex 1401 double monochromator, a cooled ITT FW-130 phototube, and a Victoreen electrometer. The excitation source was a Coherent Radiation CR-5 Argon laser. A Spectra Physics tunable dye laser was employed for wavelengths greater than 5286 Å. A polarization scrambler was fixed in the scattering path at all times. Samples were contained in 1-mm capillary tubes held perpendicular to the scattering plane. Laser power was measured at the sample position and was adjusted to maintain the same intensity for the amide I band (1650 cm^{-1}) of the protein samples. Raman shifts were determined relative to the intense ν_1 mode of ClO_4^- (936 cm^{-1}) or SO_4^{2-} (983 cm^{-1}).

The excitation profiles are reported as the ratio of the area under a band (determined by triangulation) to the area under the ClO_4^- ν_1 band. The ratios were corrected for sample self-absorption and instrumental spectral response and were normalized to unit bound metal concentration. The data for Fe(EDDHA)$^-$ are based on resolved peak heights rather than the more laboriously determinable peak areas, as these proved to be proportional, independent of wavelength. Absorption spectra were determined on a standard Cary Model 14 spectrophotometer.

Results and Discussions

Raman Spectra. Spectra of Fe(III)–transferrin, Cu(II)–transferrin, and apotransferrin solutions at similar low concentrations are compared in Figure 1. The four Raman bands discernible for dilute apotransferrin are dominant features of normal protein spectra:[24] a phenylalamine ring mode at 1004 cm^{-1}; a broad amide III band near 1325 cm^{-1}; a CH_2 deformation mode near 1450 cm^{-1}; and a strong band at 1650 cm^{-1} resulting from the superposition of an amide I mode and the H_2O deformation mode. Additional bands are seen in the Fe(III)–transferrin spectrum at 1174, 1288 (with a shoulder near 1278), 1508, and 1613 cm^{-1} (partially obscured by amide I). These have also been reported by Tomimatsu, *et al.*,[19] and by Carey and Young.[20] Each of these bands is polarized, and their intensities vary strongly with excitation wavelength (*vide infra*), showing that they are resonance enhanced.

There are no frequency shifts when $HC^{18}O_3^-$ replaces $HC^{16}O_3^-$ in the protein. None of the bands can therefore arise from carbonate vibrations. The data for

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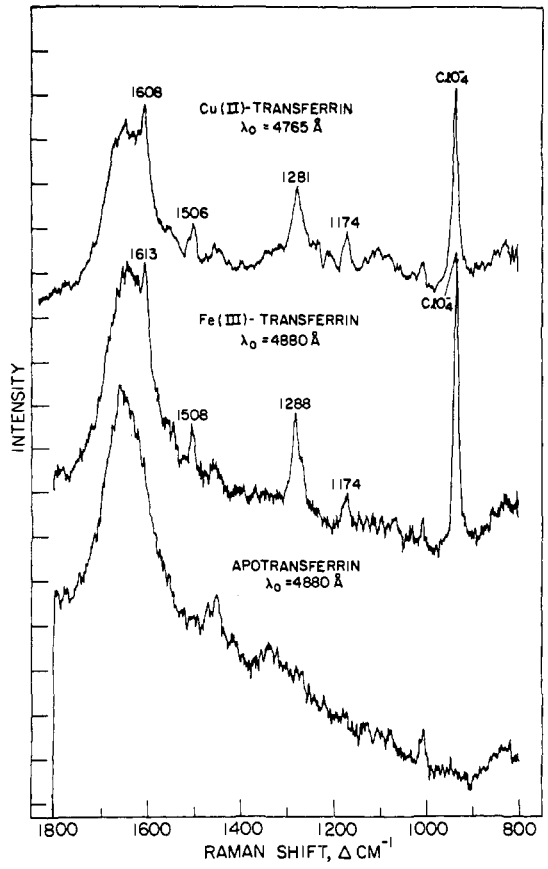

Figure 1. Raman spectra of solutions of copper(II)–transferrin iron(III)–transferrin and apo-transferrin, obtained in 1-mm capillary tubes by transverse excitation with an Ar^+ laser (100–150 mW) at the indicated wavelengths. Instrument conditions: spectral slit width 8 cm^{-1}, scan rate 50 cm^{-1}. Concentrations: 1.73×10^{-4}, 1.48×10^{-4}, and 1.50×10^{-4} M for copper–, iron–, and apo-transferrin, respectively. The metalloproteins were kept at pH 7.9 with 0.12 M tris buffer, and the solutions contained 0.05 M $NaClO_4$ added as an internal standard.

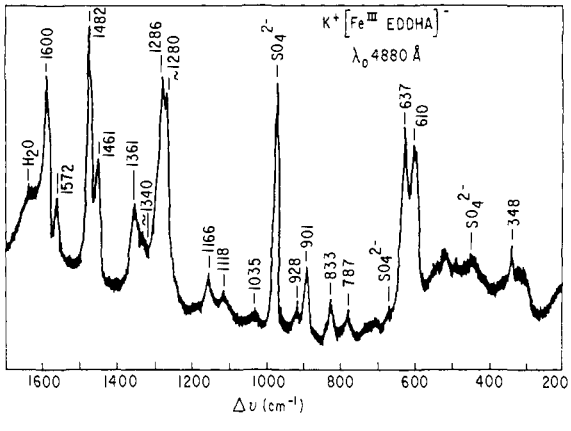

Figure 2. Raman spectrum of $K^+[Fe(EDDHA)^-]$, 2×10^{-3} M, in aqueous Na_2SO_4 (0.5 M) at 4880 Å. Instrument conditions as for Figure 1.

Cu(II)-transferrin are strikingly similar to those of the Fe(III) protein, with resonance-enhanced bands shifted slightly to lower frequency: 1608, 1506, 1281 (plus shoulder), and 1174 cm^{-1}.

The resonance Raman spectra of $Fe(EDDHA)^-$, shown in Figure 2, is at a higher concentration and is of higher quality than the protein spectra. It contains

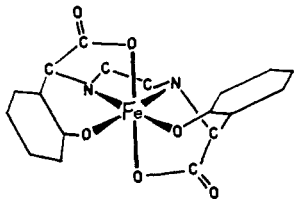

Figure 3. Proposed structure for $Fe(EDDHA)^-$. (Hydrogen atoms omitted for clarity.)

four prominent bands at about the same positions as those observed in the transferrin complexes: 1168, 1286 (with a shoulder at 1280), 1482, and 1600 cm^{-1}. Several other bands are clearly resolved in the spectrum of the model compound, notably at 1542, 1461, 1361, 901, 833, 787, 637, and 610 cm^{-1}. The ligand anion,[25] $EDDHA^{4-}$, provides six binding sites for Fe^{3+}: two amine nitrogen atoms, two carboxylate oxygen atoms, and two phenolate oxygen atoms. While the commercial acid H_4(EDDHA) is of unspecified isomeric nature, a meso and a racemic form being possible, only one Fe^{3+} complex was crystallized. Inspection of space-filling models indicates that all isomers of the complex are sterically improbable except the one shown in Figure 3, involving the racemic form of the ligand. A single isomer has also been found for the Co^{3+} complex of the sterically similar ligand ethylenediaminedisuccinic acid, for which the structure shown in Figure 3 was proposed on the basis of nmr and CD evidence.[26]

The visible absorption of Fe(III)–phenolate complexes has long been known, and the deep red color of $Fe(EDDHA)^-$ can confidently be ascribed to the interaction of the phenolate groups with the Fe^{3+} ion. Certainly the amine and carboxylate binding sites provide no basis for an intense visible absorption. It is therefore reasonable to infer that the resonance Raman bands of $Fe(EDDHA)^-$ arise from phenolate vibrations, and indeed all of them correlate with phenolate modes.[27-29] In particular the four bands which are also seen in the Fe(III)- and Cu(II)–transferrin spectra can be assigned as follows: 1168 cm^{-1}, in plane C–O bending, with substantial ring character; 1286 cm^{-1}, phenolate C–O stretching (compare alkaline phenol[29] at 1281 cm^{-1}); 1482 cm^{-1}, a symmetric ring stretch (1502 cm^{-1} in phenol); and 1600 cm^{-1}, a ring "quadrant" stretch (1614 cm^{-1} in p-cresol).

Carey and Young[20] assigned some of the resonance Raman bands of Fe(III)–transferrin to imidazole vibrations, and Tomimatsu, *et al.*,[19] also allowed for this possibility. Imidazole vibrations might be expected to shift upon deuteration, however, and we observed no shifts in D_2O. All of the metal–transferrin resonance Raman bands are assignable to tyrosine. Since the Fe(III)–transferrin electronic spectrum is remarkably similar to that of $Fe(EDDHA)^-$ (*vide infra*), it is unlikely that imidazole contributes significantly to the visible absorption. Imidazole would not therefore be

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expected to contribute to the resonance Raman spectrum, even if it is involved in Fe(III) binding.

Electronic Spectra. The electronic spectrum of Fe(EDDHA)$^-$ is shown in Figure 4. It is very similar to that of Fe(III)–transferrin.[2] Both protein and model exhibit a broad band near 475 nm, while the shoulder in the model spectrum near 315 nm has a poorly resolved counterpart in the protein spectrum.

The two bands of EDDHA^{4-} are readily assigned to the usual lowest allowed transitions of benzene derivatives. These are derived from the $^1B_{2u} \rightarrow {}^1A_{1g}$ and the $^1B_{1u} \rightarrow {}^1A_{1g}$ transitions of benzene, in order of increasing energy.[30] Their shift to higher energy upon Fe(III) binding is similar to that which occurs upon protonation and is due to σ bond formation with an oxygen lone pair and reduced conjugation with the phenyl ring. The two additional low-energy bands in the Fe(EDDHA)$^-$ spectrum are logically assigned to ligand $\rightarrow$ metal charge-transfer transitions, an assignment consistent with the weakness of ligand-field transitions, especially for high-spin Fe(III)[31] and the high reduction potential of Fe(III).

We assign the lowest energy band (475 nm) to a transition from $p\pi$ orbitals on the phenolate oxygen atoms to the half-filled $d\pi^*$ orbitals on Fe(III). Such $p\pi \rightarrow d\pi^*$ transitions, of similar energies and intensities, are well known for Fe(III) complexes of good π-donor ligands such as N_3^- [32] and NCS^-.[33] For the band at 315 nm, assignment to $p\pi \rightarrow d\sigma^*$ charge transfer is suggested by the energy gap, $\sim$11,000 cm^{-1}, separating it from the lowest energy, $p\pi \rightarrow d\pi^*$ transition. This gap is very near the $d\pi$–$d\sigma^*$ separation, $10Dq$, for Fe$(OH_2)_6^{3+}$.[31]

The high intensity of the charge-transfer bands is likely due to mixing with low-lying phenyl $\pi \rightarrow \pi^*$ transitions, which have appropriate (long axis polarized) symmetry. Similar intensity enhancement is available to N_3^- and NCS^-, which have low-lying $\pi \rightarrow \pi^*$ transitions. While charge-transfer transitions from acetate or hydroxide $p\pi$ orbitals to Fe(III) should presumably lie in the visible or near-ultraviolet region, they are not in fact observed for mononuclear complexes, such as Fe(EDTA)(OH)$^{2-}$.[34] Apparently the overlap between donor and acceptor orbitals is too small to allow appreciable intensity for the transition, in the absence of mixing with available low-energy $\pi \rightarrow \pi^*$ transitions.

The $p\pi \rightarrow d\pi^*$ assignment for the $\sim$475-nm band of Fe(EDDHA)$^-$ is consistent with the observation[35] that complexation of Fe(III) by successive phenolate groups produces a blue shift in the visible absorption maximum by about 2000 cm^{-1} per phenolate group. Interaction with an increasing number of phenolate oxygen $p\pi$ electrons would be expected to raise the energy of the antibonding $d\pi^*$ orbitals. The observation that the energy of this band is essentially the same for Fe(III)–transferrin (λ_{max} 470 nm) as for Fe(EDDHA)$^-$ (λ_{max} 475 nm) is therefore strong evidence that the number of bound phenolate groups is the same, namely two. The absorptivity of the visible band is roughly additive for successive phenolate binding, amounting to 1–2 $\times$ 10^3 M^{-1} cm^{-1} per phenolate ligand.[35] The absorptivities of Fe(III)–transferrin[2] (2.5 $\times$ 10^3 M^{-1} cm^{-1}) and Fe(EDDHA)$^-$ (4.0 $\times$ 10^3 M^{-1} cm^{-1}) are consistent with the binding of two phenolate groups.

Cu(II)–transferrin displays a visible absorption band[12] of similar intensity and slightly higher energy (λ_{max} 440 nm) than that of Fe(III)–transferrin. It seems likely that the assignment should again be ligand $\rightarrow$ metal charge transfer, this time $p\pi \rightarrow p\sigma^*$, since Cu(II) has but one half-filled d orbital. (An alternate possibility is $d\sigma^* \rightarrow \pi^*$, metal $\rightarrow$ ligand charge transfer.) Intense absorption has been observed for planar four-coordinate Cu(II) complexes with two cis-coordinated trichlorophenolate ligands.[36] We found that Cu(EDDHA)$^{2-}$, prepared by the same technique as used for Fe(EDDHA)$^-$, is a normal blue Cu(II) complex and does not show an intense charge-transfer band. Presumably the normal tetragonal Cu(II) coordination geometry is maintained by equatorial binding of the amine and carboxylate groups, leaving only weak interactions, if any, with the phenolate groups at the axial sites. The inference is that the transferrin binding site, maintaining its own structural constraints, imposes equatorial phenolate binding on Cu(II).

Resonance Raman Enhancement. The Raman modes which are expected to be enhanced upon excitation in an absorption band are those which couple appreciably to the electronic transition; *i.e.*, the movement of the nuclei in these normal modes significantly alters the electronic transition probability.[37] The intensity pattern in the resonance Raman spectra of Fe(EDDHA)$^-$ and Fe(III)– and Cu(II)–transferrin can be understood qualitatively on this basis. If, as described above, the resonant electronic transition involves $p\pi^* \rightarrow d\pi^*$ charge transfer, then the required depopulation of the oxygen $p\pi$ orbital would lower the extent of conjugation with the π system of the phenyl ring; *i.e.*, the contribution of quinoid resonance forms for the phenolate ion would decrease. The vibrations most strongly involved would be C–O stretching and ring stretching modes, and these are in fact the strongest bands in the resonance Raman spectra, at $\sim$1280 and at $\sim$1500 and $\sim$1600 cm^{-1}, respectively.

Aside from the many weak features which are evident in the better resolved Fe(EDDHA)$^-$ spectrum, the main spectral difference between model and proteins is the change in relative intensity for in plane deformation modes. In Fe(EDDHA)$^-$ the ring deformation modes[21] at 637 and 610 cm^{-1} are quite strong. These are unobserved for Fe(III)– and Cu(II)–transferrin, which, however, show greater relative intensity for the C–O in-plane bending mode, at 1174 cm^{-1}. This difference may arise from the fact the phenolate groups in EDDHA^{4-} are ortho while those in the protein (tyrosine) are para substituted.

We had at first expected that metal–oxygen stretching modes, which should occur near 400 cm^{-1}, would be enhanced in a phenolate $\rightarrow$ metal charge-transfer band.

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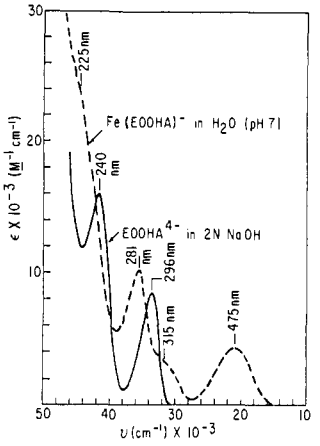

Figure 4. Absorption spectra of $EDDHA^{4-}$ and $Fe(EDDHA)^-$.

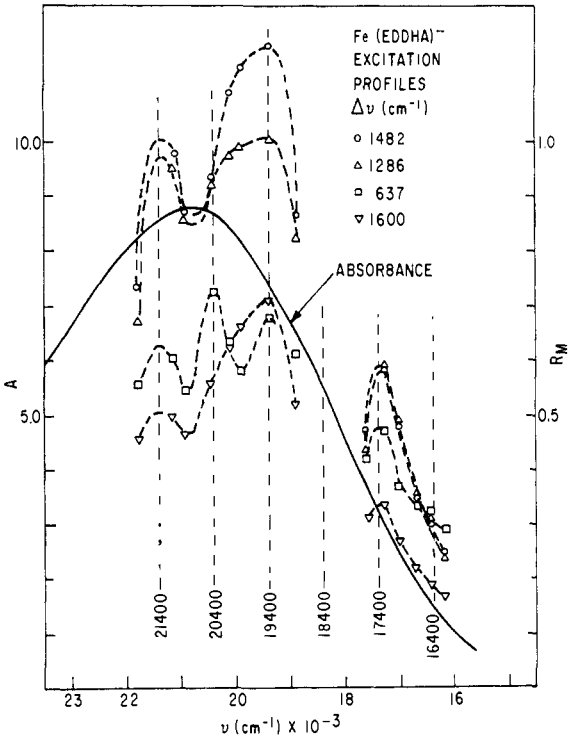

Figure 5. Raman excitation profiles and absorption spectrum of $Fe(EDDHA)^-$. The relative molar scattered intensity R_M is the intensity ratio for the band in question to the 983-cm^{-1} (ν_1) band of the SO_4^{2-} internal standard, corrected for sample self-absorption and instrument spectral response.

A careful search of the Fe(III)–transferrin spectra revealed only weak, broad emission below 800 cm^{-1}. In the $Fe(EDDHA)^-$ spectrum (Figure 2) this region is better resolved, and Fe–O stretching may contribute to the complex feature found near 400 cm^{-1}, but resonance enhancement, if present at all, is certainly not impressive. This negative finding may be understood, however, on the basis of the $p\pi \rightarrow d\pi^*$ assignment of the resonant electronic transition. The $d\pi^*$ orbitals do not interact strongly with the ligands, and adding an electron to them would not significantly alter the Fe–O bonding.

Raman Excitation Profiles. It is possible to probe the vibrational–electronic coupling which gives rise to resonance enhancement by monitoring the Raman intensities as a function of the excitation wavelength.

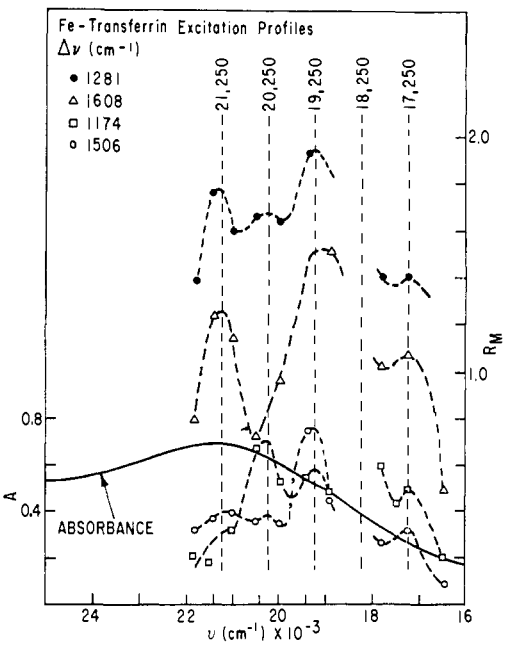

Figure 6. Raman excitation profiles and absorption spectrum of Fe(III)–transferrin. As in Figure 5, but the internal standard is ClO_4^- (ν_1 = 936 cm^{-1}).

In the case of $Fe(EDDHA)^-$ and Fe(III)–transferrin, much of the visible absorption band could be covered with available Ar^+ laser lines, 458–529 nm, and a rhodamine 6 G tunable dye laser, 560–630 nm. The resulting excitation profiles are shown in Figures 5 and 6. They are remarkable, and in fact unprecedented, in their complexity.

In the few cases where Raman intensities have been monitored across absorption bands, *e.g.*, heme proteins[38,39] and MnO_4^-,[40] the excitation profiles are well behaved, following the general contours of the absorption band. There are reported instances, naphthalene,[41] retinal,[41] and rhodopsin,[42] where the excitation profile shows a peak at energies somewhat below an intense absorption band. These have been attributed to resonance with forbidden transitions, either triplet or singlet in character, although the mechanism for resonance with a forbidden transition is unclear. Transferrin and $Fe(EDDHA)^-$ show still more complicated behavior. In this case the strong visible absorption band is clearly responsible for resonance enhancement. It is very broad, but shows no sign of splitting. (We have recorded absorption spectra of Fe(III)–transferrin down to liquid helium temperature and observed a slight narrowing due to loss of hot-band contributions, but no development of structure.) The excitation profiles are highly structured, however, with more than one maximum apparent.

For each of the strong Raman bands monitored, the first intensity maximum occurs in the tunable laser range, in the low energy tail of the absorption band. At higher energies the individual profiles vary in shape,

(38) T. G. Spiro and T. C. Strekas, *Proc. Nat. Acad. Sci. U. S.*, 69, 2622 (1972).
(39) T. C. Strekas and T. G. Spiro, *J. Raman Spectrosc.*, 1, 387 (1973).
(40) W. Kiefer and H. J. Bernstein, *Mol. Phys.*, 23, 385 (1972).
(41) L. Rimai, M. E. Hyde, H. C. Heller, and D. Gill, *Chem. Phys. Lett.*, 10, 207 (1971).
(42) A. Lewis, *J. Raman Spectrosc.*, 1, 473 (1973).

but in every case there are at least two more maxima, occurring at 2000 and 4000 cm^{-1} above the first maximum. In the case of the in-plane deformation mode, 637 cm^{-1} for Fe(EDDHA)$^-$ and 1174 cm^{-1} for Fe(III)–transferrin, a fourth pronounced maximum appears 3000 cm^{-1} above the first maximum, while the remaining profiles show weak maxima or shoulders at this position. The dashed vertical lines in Figures 5 and 6 illustrate quite clearly that the excitation profile maxima constitute a uniform progression with a 1000-cm^{-1} spacing. We attribute this pattern to resonance Raman scattering from a series of *excited state vibrational levels* of this spacing. The likeliest candidate for the resonant excited state vibration is the C–O stretching mode. Since as described above the electronic transition depopulates the oxygen $p\pi$ orbital, thereby reducing conjugation to the phenyl ring, the C–O bond length would be expected to increase significantly in the excited state. Indeed lengthening of the C–O bond is no doubt the major structure change in the excited state and a dominant Frank–Condon progression in the C–O stretching vibration would be expected. The frequency of this vibration should decrease appreciably from its ground state value, $\sim$1280 cm^{-1}. A value of $\sim$1000 cm^{-1} is reasonable for a C–O single bond. Other vibrations, associated with structural features which do not change as much in the excited states, should show much weaker Frank–Condon progressions; they may form subprogressions on the dominant ν_{C-O} levels.

If this interpretation is correct then the first excitation profile maximum corresponds to the origin (0–0) of the electronic transition, 17,400 cm^{-1} for Fe(EDDHA)$^-$ and 17,250 cm^{-1} for Fe(III)–transferrin, there being no additional maxima at lower energy. The higher energy maxima correspond to 0–2, 0–3, and 0–4 transitions to vibrational levels involving C–O stretching. Unfortunately there is a gap in the available laser frequencies at the predicted 0–1 position. The maximum of the absorption band is found between three and four quanta of the C–O stretching vibration, where the Frank–Condon overlaps are presumably largest.

Inspection of the excitation profile shows that this description is oversimplified. Only the in plane deformation modes show well resolved maxima at 0–3. For the three ring and C–O-stretching vibrations a weak maximum or shoulder is found at this position. There appears to be additional intensity filling in the valley between 0–2 and 0–3. This may represent an influence of additional vibronic levels, involving other vibrations than C–O stretching. Or it might possibly arise from a new electronic origin; the $p\pi$–$d\pi^*$ transition might be split by interaction between the two phenolate groups.

While these details remain to be resolved, it is apparent that Raman excitation profiles exhibit greater selectivity for excited state levels than does the absorption spectrum, and they provide a potentially powerful approach to vibronic spectroscopy.

Conclusion

1. The resonance Raman spectra of Fe(III)– and Cu(II)–transferrin are completely assignable to enhanced phenolate (tyrosine) vibrations as demonstrated by the model compound Fe(EDDHA)$^-$. Neither imidazole nor bicarbonate participate significantly in the chromophoric unit, although they may be bound to the metal ion.

2. The visible absorption band of Fe(EDDHA)$^-$ and Fe(III)–transferrin is assigned to a charge-transfer transition from the oxygen $p\pi$ orbital of bound phenolate to the Fe(III) $d\pi^*$ orbitals and probably to the Cu(II) $d\sigma^*$ orbital in Cu(II)–transferrin. The close similarity of the Fe(EDDHA)$^-$ and Fe(III)–transferrin visible absorption bands implies that the transferrin binding site involves two tyrosines. The strongly enhanced Raman modes correspond to those vibrations, C–O stretching, ring stretching, and in plane deformation, which should be most strongly vibronically involved in the electronic transition.

3. Since bicarbonate is required for the development of the Fe(III)–transferrin chromophore, it must induce a protein conformation change which allows binding of Fe(III) by two tyrosine phenolate groups. Whether it is also bound directly to the iron atom remains an open question.

4. The Raman excitation profiles show unprecedentedly complex behavior, within which a progression of peaks with a uniform 1000-cm^{-1} spacing can be discerned. This is interpreted as reflecting enhanced Raman scattering from successive excited state vibrational levels involving the C–O stretching mode.

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