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Correlations between Reduction Potential, CO Stretch Frequency, and CO Half-Bandwidth in Hemoproteins†

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ABSTRACT: For 15 natural and artificial hemoproteins the Fe(III)/Fe(II) reduction potentials (E_{m7}) correlate linearily with the CO stretch frequencies (ν_{CO}) and inversely with the half-bandwidths ($\Delta\nu_{1/2}$) of the carbonyl derivatives. Deviations from the correlation E_{m7}/ν_{CO} do occur as a sign of additional factors affecting ν_{CO}. Thus, an aberrantly low ν_{CO} indicates hydrogen bonding of CO whereas aberrantly high values of four hemoproteins are discussed in terms of steric restraints upon CO. Horseradish peroxidase isoenzyme C was reconstituted with three and whale myoglobin with four artificial hemes, substituted at the 2,4-positions. For both sets of artificial hemoproteins, ν_{CO} responds to E_{m7} in the same manner as for native hemoproteins. In the case of $\Delta\nu_{1/2}$, the covariation with E_{m7} was less pronounced after substitution. Quantum mechanical Hartree–Fock–Slater-type calculations on a carbonylheme model ($N_5C_{14}Fe$·CO) show that ν_{CO} and $\Delta\nu_{1/2}$ respond to changes in total charge on carbonylheme, simulated by adding or withdrawing electrons from this model. The experimental correlations between ν_{CO} and $\Delta\nu_{1/2}$ with E_{m7} are accounted for by a high density of states for metal-bound CO around the highest occupied and lowest unoccupied orbitals. The hydrogen bonding to CO found only with proteins of low E_{m7} can be explained by the increase of negative charge upon the oxygen of CO with increasing negative charge upon carbonylheme. A pronounced decrease of $\Delta\nu_{1/2}$ was calculated for a Fe–C–O unit bent at 135° and a smaller decrease for a unit tilted 45° from heme normal. The increase in $\Delta\nu_{1/2}$ that accompanies an increase in space available to liganded CO and increased conformational randomness is discussed in terms of electron distribution and E_m.

In hemoproteins, the iron atom, porphyrin, protein moiety, and surrounding solvent form a balanced system that can engage in a variety of reactions with the transfer of two, one, or zero electrons. Most of these proteins carry protoheme; others carry heme A or C. The Fe(III)/Fe(II) reduction potential E_m[1] reflects the electronic structure of the iron atom and is hence a highly informative parameter. The E_m values of protohemoproteins depend upon the protein moiety and span a range of about 0.5 V. Iron spin state and axial ligand strength may determine E_m and the reactivity of a hemoprotein (Williams, 1961; Perutz, 1972; Moore & Williams, 1977), but this idea has met difficulties (Korszun et al., 1982). Another proposal is that E_m is determined by the dielectric constant of the heme surroundings (Kassner, 1972). This proposal has been examined by Stellwagen (1978), who emphasized the importance of the extent of porphyrin exposure to solvent water. The replacement of the 2,4-vinyl groups of protoheme by other electron-redistributing groups affects not only E_m of the hemoprotein (Yamada et al., 1975) but also biological functions such as the binding of O_2 to Hb and Mb (Makino & Yamazaki, 1974; Asakura et al., 1982) or the rate of reduction of peroxidase compound II by electron donors (Chance & Paul, 1960; Ohlsson & Paul, 1973).

Bound CO has extensively been used to probe the reactivity of metal atoms in copper proteins and hemoproteins and on metal surfaces, changes concerning CO being monitored by means of various vibrational spectroscopic methods. A compilation of values for E_{m7} and ν_{CO} of five protohemoproteins and cytochrome a_3 indicated a linear covariation between E_{m7} and ν_{CO} (Barlow et al., 1976). This study also showed that deviations from the linear correlation did occur in carbonyl-peroxidases and attributed them to the formation of a hydrogen bond between CO and the protein moiety. More limited correlations have been suggested between ν_{CO} and porphyrin pK_3 and ν_{NH} (Alben & Caughey, 1968), between ν_{CO} and n log $P_{1/2}$ for O_2 binding (Berzofsky et al., 1972), and between

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[1] Abbreviations: Mb, myoglobin; Hb, hemoglobin; CTT, *Chironomous thummi thummi*; CCP, cytochrome *c* peroxidase; HRP, horseradish peroxidase; peroxidase, EC 1.11.1.7; IR, infrared; NMR, nuclear magnetic resonance; ν_{CO}, stretch frequency of CO IR absorption band; $\Delta\nu_{1/2}$, half-bandwidth of CO IR absorption band; E_m, reduction potential; E_{m7}, E_m at pH 7; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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ν_{CO} and integrated absorbtivity (Alben et al., 1981). The correlation between ν_{CO} and integrated absorptivity is somewhat controversial (Satterlee et al., 1978).

Another approach to the understanding of hemoprotein functions is to evaluate electronic structures from quantum chemical calculations. Porphyrin "π"orbitals were well described in a free-electron model (Gouterman, 1978). Extended Hückel (EH) calculations [Zerner et al. (1966) and references cited therein] revealed the characteristics of iron compared to other transition metals, and a similar study was performed on an ab initio level (Ohno, 1980). Extensive INDO (intermediate neglect of differential overlap) and EH calculations on hemes have been presented (Loew & Kirchner, 1978; Herman et al., 1980; Waleh & Loew, 1982), including electronic excitations, photodissociation, and bent, tilted, or kinked geometries of bound CO. A recent study applied the so-called Xα multiple scattering scheme to iron porphin without axial ligands with special attention to iron 4p orbitals in the porphin plane (Sontum et al., 1983).

The present investigation approaches experimentally and theoretically the relationship between E_m, ν_{CO}, and the half-bandwidth, $\Delta\nu_{1/2}$, of the CO stretch band for a variety of protohemoproteins and cytochrome a_3. We have also measured E_{m7} and ν_{CO} of a peroxidase and a myoglobin as functions of 2,4-porphyrin substitutions. We have experimentally found that $\Delta\nu_{1/2}$ is closely correlated to E_m and that ν_{CO} of carbonyl hemoproteins largely is a function of E_m, with deviations from linearity being attributed to ligand geometric distortion or hydrogen bonding. The electronic structure of carbonylheme has been modeled by quantum mechanical calculations to quantitatively dissect on the atomic level the problem of CO as an isolated vibrator. We use the Xα potential in a linear combination of atomic orbitals (LCAO) scheme different from the muffin tin procedure in Hartree–Fock–Slater (HFS) multiple scattering calculations (Case et al., 1979). The correlations between E_m, ν_{CO}, and $\Delta\nu_{1/2}$ can be modeled by these calculations, which show that the iron 4p orbitals play the dominant role in transferring perturbations of the electron density from porphyrin to ligand.

Materials and Methods

Hemins. The dimethyl esters of deuteroporphyrin (2,4-H) IX, mesoporphyrin (2,4-diethyl) IX, and 2,4-diacetyldeuteroporphyrin IX were prepared according to established procedures (Caughey et al., 1966). Purities were confirmed by means of optical spectra and melting points. The esters were saponified in 6 M HCl, and iron was inserted into the porphyrins according to Falk (1964). The preparation of 2,4-dibutenylheme has been described (Ohlsson & Paul, 1973). The hemes were checked for authenticity by visible spectroscopy of the pyridine hemochromes and by IR spectroscopy of a 1% mixture in KBr. A plateau at 600–650 nm in the hemochrome spectrum disqualified a heme preparation from use. For this reason, several 2,4-diformylheme preparations were discarded in spite of excellent qualities of the corresponding ester or free porphyrin.

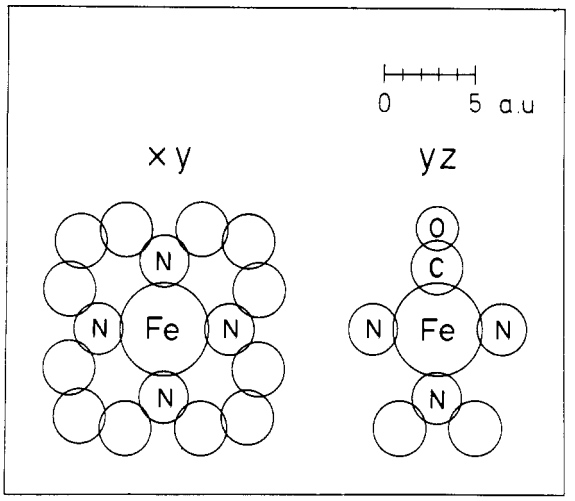
Hemoproteins. Sperm whale myoglobin (Sigma type II) was purified on CM-52, and component IV (Rothgeb & Gurd, 1978) was collected. Horseradish peroxidase fractions A2 and C2 were prepared from the root (Paul & Stigbrand, 1970) and rechromatographed until homogeneous by gel electrophoresis. Hemes were removed by means of acid acetone (Mb; Rothgeb & Gurd, 1978) or acid butanone (HRP; Teale, 1959). Apoproteins were stored in liquid nitrogen until used. Proteins were reconstituted with artificial hemins in 10 mM Tris, pH 8.0 (Ohlsson & Paul, 1973), a procedure recently (Ahmad & Kincaid, 1983) found to give a minimum of heme disorder (La Mar et al., 1980). After dialysis against 10 mM potassium phosphate–1 mM EDTA, pH 6.0, the reconstituted proteins were chromatographed on CM-52 for Mb and on DE-52 for HRPC. A linear gradient from the above buffer to 150 mM potassium phosphate–1 mM EDTA, pH 6.0, eluted the holoprotein as a single component. Leghemoglobin (Ellfolk, 1960) and lactoperoxidase (Paul et al., 1980) were isolated as described. Cytochrome *c* peroxidase was a gift from Dr. T. Yonetani. Holohemoproteins were dialyzed against 50 mM phosphate–1 mM EDTA, pH 7.0, concentrated by ultrafiltration under nitrogen, rapidly frozen, and stored in liquid nitrogen.

E_{m7} Measurements. Reduction potentials of the hemoproteins were determined optically in a cell described elsewhere (Ohlsson & Paul, 1983) from equilibria with oxidation–reduction indicators. The protein–indicator solution was deaerated by means of argon bubbling for 3 h at a flow rate of 30 mL/Hour. A very small volume of sodium dithionite in 10 mM borate buffer, pH 9, was delivered in portions to the solution from a gas-tight microsyringe. Thionine (Merck, Darmstadt), E_{m7} = 0.056 V (Clark, 1960), toluylene blue (Merck), E_{m7} = 0.110 V (Clark, 1960), and phthiocol (K & K, New York), E_{m7} = −0.168 V (Ohlsson & Paul, 1982), were used as indicators in concentrations that gave absorbance changes about equal to those of the hemoprotein (40–60 μM) upon reduction. Spectra over the range 450–700 nm were scanned at 25 °C in a Beckman DU-7 or Acta III spectrophotometer. Whale myoglobin gave E_{m7} = 0.043 V at 25 °C as compared to the literature value of 0.050 V at 30 °C (Brunori et al., 1971).

Infrared Measurements. Prior to IR measurements, proteins were concentrated by ultrafiltration under nitrogen gas and then diluted 10–15-fold with deuterated buffer, "pH" 7.0 as read on the pH meter; this procedure was repeated twice. After concentration to 2 to 5 mM, the solution was stirred under a stream of scrubbed (chromic perchlorate–amalgamated Zn) CO for 30 min, reduced by the addition of a small quantity of fresh dithionite, and injected into a Beckman-RIIC variable-temperature, 0.2-mm IR cell, fitted with ZnS windows and thermostated at 20 °C. Sample integrity was checked after infrared analysis by optical spectrophotometry directly of the IR cell. The IR spectra were recorded (15 000 scans each) with a Digilab FTS-14C interferometer on a single-beam mode at 1-cm^{-1} resolution. Separate spectra (15 000 scans each) of buffer and air were recorded to permit conversion to absorbance. Unless otherwise noted, spectra were not smoothed for presentation. Samples of human Hb·CO were examined from time to time by using the CO stretch maximum at 1951.5 cm^{-1} in H_2O or ^2H_2O as a check of instrument accuracy.

The IR absorption bands are presented as obtained, with the following comments. An important factor in determining ν_{CO} of either a P or H band (for symbols, see below) is often the shape of the absorbance itself. While the shape may be greatly distorted by use of the computerized base-line subtraction routines available with this interferometer, it is our experience that ν_{CO} cannot be shifted more than 0.5 cm^{-1} without creating base lines with ridiculous slopes and/or curvatures. Broad H bands such as those observed for HRP·CO do not allow determination of ν_{CO} or $\Delta\nu_{1/2}$ to better than 1 cm^{-1}. Else, $\Delta\nu_{1/2}$ is relatively independent of computor manipulation so that this value is reproducible to within 1 cm^{-1}. $\Delta\nu_{1/2}$ for Mb·CO's were estimated by doubling $(1/2)\Delta\nu_{1/2}$ as determined from the high-energy side of the P band; this band

Chart I: Geometry of Porphyrin Carbonyl Model



is Gaussian (Caughey et al., 1981).

Instruments and Chemicals. Commercial Ar (AGA, Stockholm) was purified by passage through an Oxy-Trap system (Alltech Assoc., IL) to give $O_2 < 0.1$ ppm. Fresh sodium dithionite (RV Chemicals, Cheshire, England) was divided under Ar into 2-mL tubes, sealed, and stored over desiccant at −20 °C until use. Deuterium oxide (99.8% ^2H), $^2H_3PO_4$, and NaO^2H for deuterium buffers were purchased from Stohler Isotopes, Waltham, MA. Other chemicals were analytical grade. Water was bidistilled in glass vessels. Electronic spectra were obtained on Beckman ACTA III or DU-7 spectrophotometers, which were routinely checked with holmium oxide filter for wavelength accuracy and potassium dichromate in 5 mM H_2SO_4 for linearity. Unless otherwise stated, measurements were performed at 25 °C in 50 mM sodium phosphate–1 mM EDTA, at pH 7.0.

Calculations. One-electron equations were solved within a Hartree–Fock–Slater (HFS) LCAO approximation. The molecular potential was determined in successive iterations from Mulliken gross orbital occupation numbers, spherically averaged around each atom (Rosén et al., 1976). Numerical atomic basis functions were generated in the Fe $3d^64s^24p^0$, O $2s^22p^4$, N $2s^22p^3$, and C $2s^22p^2$ configurations by an atomic HFS program. The calculations were performed within a spin-degenerate scheme and presumed a low-spin configuration corresponding to hexacoordinated Fe(II) in a strong ligand field. Our analyses are based solely upon population matrices, and we concentrate on trends rather than on absolute numbers. The CO vibrational frequency is assumed to be proportional to the square root of the number of bonding π electrons (Paul & Rosén, 1984).

If possible changes in the solvent are ignored, shifts of E_m correspond to shifts of the ionization potential (Gerischer & Ekardt, 1983) and may thus be seen as a change in position of the Fermi edge, E_F, i.e., the mean energy of the highest occupied and lowest unoccupied molecular orbitals with respect to the vacuum level. Our heme model is a $N_5C_{14}Fe$·CO cluster that includes all atoms nearest and second nearest to iron with the ring closed by methine bridges (Chart I). The cluster will only incompletely represent heme, but we believe that basic characteristics of the Fe–C–O bonds are reproduced (Paul et al., 1983). We model altered electron availability by supplying or withdrawing electrons from the cluster. This will influence the filling of CO electron states since liganded CO has a high density of π states (DOS) around E_F (J. Paul, M. L. Smith, and K. G. Paul, unpublished results). Hence, shifts in ν_{CO}, but not the absolute position of the CO vibrational energy, due

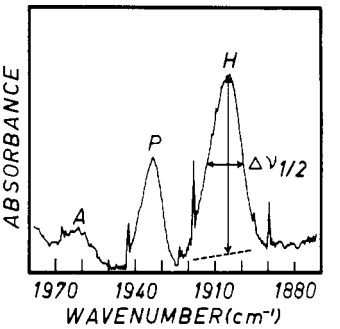


FIGURE 1: Infrared spectrum of HRPC·CO in ^2H_2O at "pH" 7.0, 50 mM sodium phosphate–1 mM EDTA, 20 °C. The CO stretch of the unperturbed vibrator is labeled P (principle), that of the hydrogen bonded species H, and that of adventitiously bound heme A.

to changes in E_m or charge may be evaluated from the CO π bond order (Paul & Rosén, 1984). A H-bond effect is found when the CO DOS is lowered in energy by an "applied" electrostatic field (Holloway & Nørskov, 1984; Paul & Rosén, 1984). In both situations, related shifts of vibrational frequencies mirror changes in the metal–carbon and the carbon–oxygen force constants. The eigenfrequencies of the coupled M–C–O vibrator can also shift as a result of a nonlinear geometry (Yu et al., 1983; J. Paul, M. L. Smith, and K. G. Paul, unpublished results). CO generally adopts a position normal to the heme plane (Jameson & Ibers, 1983). We have modeled off-normal bonding by a tilted or bent geometry, both with the C–O bond leant 45° over a methine carbon with the Fe–C and C–O bond lengths kept constant. Tilting or bending will reduce the cluster symmetry from C_{2v} to C_S.

As experimentally found (cf. below), $\Delta\nu_{1/2}$ and ν_{CO} correlate well with E_{m7} and, hence, with each other, albeit in an inverse manner. This correlation as well as the short lifetimes of about 10^{-12} s, reflected by $\Delta\nu_{1/2}$, closely resembles what is found for CO bound to metal atoms embedded in a metal surface (Hoffman, 1983; J. Paul, M. L. Smith, and K. G. Paul, unpublished results). Inhomogeneous broadening will to some extent obscure the general trend in both cases. For surface-bound CO, the homogeneous part represents energy dissipation from the vibrationally excited molecule to a nonadiabatically slightly perturbed electronic state of the absorbent (Persson & Persson, 1980; Ueba, 1982). Despite the absence of a continuous DOS at E_F in hemes, we adopt this model that correlates $\Delta\nu_{1/2}$ to the square of the charge δn oscillating to and from CO during the vibrational motion. δn is taken as the difference between the net charges on CO for $R_{CO} = R^0_{CO} \pm \Delta R_{CO}$. The CO center of mass is kept constant, and ΔR_{CO} equals 0.08 au, the mean absolute value of the vibrational amplitude $(\pi^{-2}[2h/(\mu\nu)])^{1/2}$.

Results

Figure 1 introduces CO stretch maximum (ν_{CO}) and width at half-maximal height ($\Delta\nu_{1/2}$), examplified by the spectrum of HRP·CO. The CO stretch at 1933 cm^{-1} is labeled P, for principal absorbance, and the stretch at 1903 cm^{-1} H, for hydrogen bonded. The H-band is ^1H_2O–^2H_2O-dependent (Smith et al., 1983), which is evidence for hydrogen bonding to CO, more precisely to its oxygen atom (Paul & Rosén, 1984). In the case of HRP·CO both bands are non-Gaussian and slightly asymmetric. Another band, smaller, broader, and more variable in size, appears at ~1970 cm^{-1} in some diacetyl- and dibutenylheme Mb·CO spectra and at 1960–1962 cm^{-1} in the meso- and deuterioheme Mb·CO spectra. There is no regular ratio between this band and the P or H bands. He-

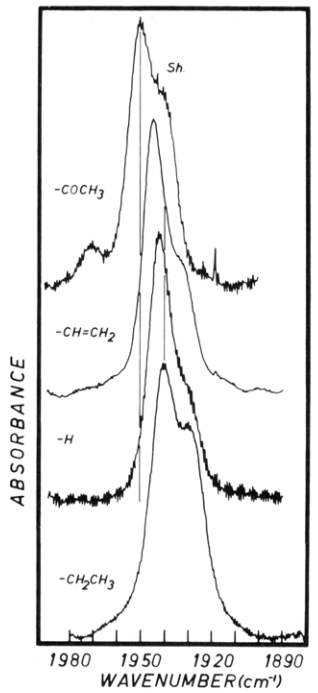


FIGURE 2: Infrared spectra of the carbonyl derivative of substituted sperm whale myoglobin IV (2–5 mM heme) at "pH" 7.0, 50 mM sodium phosphate–1 mM EDTA, 20 °C. $\Delta\nu_{1/2}$ is double $(1/2)\Delta\nu_{1/2}$ as measured on the high-energy side because of the appearance of the shoulder (Sh) at lower energy. The structure of the 2,4-substituent of the porphyrin is written at the left of the corresponding myoglobin spectrum.

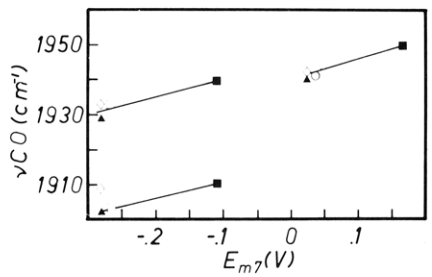


FIGURE 3: ν_{CO} of 2,4-disubstituted hemoproteins vs. E_{m7}: substituted myoglobins, top right; the P band of substituted $HRPC_2$, top left; the H band of substituted $HRPC_2$, lower left; (■) diacetyl; (□) proto; (◊) deutero; (▲) meso; (○) dibutenyl.

moproteins can spontaneously release their heme (Smith et al., 1982), and it may be relevant that artificial hemoproteins do so more easily than protohemoproteins do (Smith, 1984). The heme release from native hemoproteins is sometimes biphasic with a minor rapid phase. Native as well as reconstituted hemoproteins may contain a small proportion of heme, bound in a different manner, adventitiously bound heme. We attribute CO stretches in the region >1960 cm^{-1} to such heme and label this CO stretch A band.

In an earlier study (Barlow et al., 1976) HRPA·CO exhibited three CO stretch absorption bands (2 P and 1 H), disregarding a possibly occurring A band. Heme release and gel electrophoresis studies have subsequently disclosed two main fractions in that kind of preparation, and homogeneous HRPA, like HRPC, exhibits only two bands with pH-dependent proportions.

The IR spectra of sperm whale Mb·CO and four artificial, heme-substituted carbonylmyoglobins look rather similar in overall shapes with low-energy shoulders (Figure 2; dibutenyl-Mb·CO is closely similar to Mb·CO). The position of νCO shifts smoothly from 1940 cm^{-1} for mesoheme Mb·CO

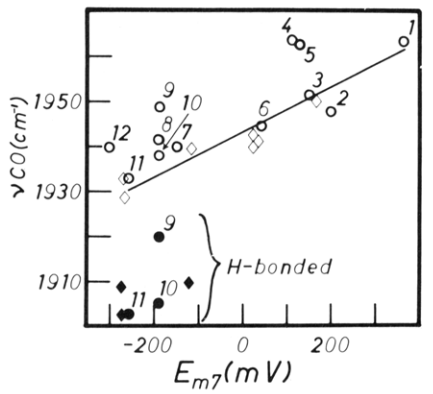


FIGURE 4: Experimental values of ν_{CO} vs. emf for hemoproteins: (○ and ●) native proteins; (◊ and ♦) artificial peroxidases and myoglobins. Cytochrome a_3 (1), leghemoglobin (2), human hemoglobin A (3), cytochrome *o* (4), *Chironomous thummi thummi* hemoglobin (5), sperm whale myoglobin (6), cytochrome P-450_{cam} (7), lactoperoxidase (8), cytochrome *c* peroxidase (9), horseradish peroxidase A_2 (10), horseradish peroxidase C_2 (11), and cytochrome P-450 (12). The drawn line obeys the relationship $\nu_{CO} = 1943 + 55E_{m7}$ (in volts).

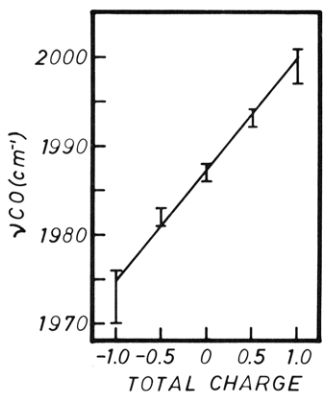


FIGURE 5: Calculated ν_{CO} of $N_5C_{14}Fe$·CO vs. total charge upon complex. A more negative cluster mimics a lower E_m.

to 1950 cm^{-1} for 2,4-diacetylheme Mb·CO. (See paragraph at end of paper regarding supplementary material.)

Figure 3 gives ν_{CO} vs. E_{m7} for HRPC and Mb reconstituted with 2,4-disubstituted hemes. Three or four E_{m7}/ν_{CO} values form a cluster with the value of the diacetyl substitute well apart. The slope between cluster and outlier is within experimental error the same for HRPC and Mb, 53 and 56 cm^{-1} V^{-1}, respectively.

CO Stretch Frequency, ν_{CO}. In Table I and Figure 4 we have gathered available data concerning carbonyl infrared spectra and E_{m7} values of various natural, and 2,4-disubstituted HRPC and Mb. The straight line was drawn through the collection of those P-band values considered to be unperturbed. The nature of perturbing effects will be discussed later. The slope of the line is 55 cm^{-1} V^{-1}, equal to those of the two substituted hemoproteins in Figure 3. We have not included hemoglobins reconstituted with 2,4-disubstituted hemes (Wallace et al., 1982) since the reduction potentials may be clouded by the concomitant loss of cooperativity in artificially substituted hemoglobins (Seybert et al., 1977; Asakura & Sono, 1974). The infrared data from various laboratories had been obtained under different buffer conditions at temperatures ranging from 0 to 35 °C, values at 25 °C or closest available temperature being presented in Table I. The position and width of the P band of Mb·CO change very little from 8 to 25 °C at constant pH (Caughey et al., 1981).

The calculated dependence of ν_{CO} upon total charge is shown in Figure 5 for the $N_5C_{14}Fe$·CO model. As a result of in-

Table I: Reduction Potentials in the Absence of Exogenous Ligands and ν_{CO} and $\Delta\nu_{1/2}$ for Carbonyl Hemoproteins at pH 7.0

protein	E_{m7} (mV)[a]	ref	ν_{CO}[b]	$\Delta\nu_{1/2}$	ref
$HRPC_2$	−250	*c*	1933 (1903)	9.5 (14)	*d*
meso-$HRPC_2$	−266	*c*	1929 (1903)	11 (17.5)	*d*
deutero-$HRPC_2$	−265	*c*	1933 (1909)	9 (19)	*d*
diacetyl-$HRPC_2$	−117	*c*	1940 (1910)	12 (15.5)	*d*
$HRPA_2$	−196 ± 3	*x*	1939 (1905)	12 (10)	*d*
cyt *c* peroxidase	−194	*e*	1949 (1922)	(13)	*d, f, g*
lactoperoxidase	−191 ± 2	*h*	1941 (1955)	13	*d*
cytochrome P-450	−303	*i*	1941	12–16	*j*
P-450 + camphor	−173	*i*	1940	12.5	*j*
myoglobin IV	43 ± 3	*x*	1944.5	9.5	*d, k*
meso-Mb	23 ± 3	*x*	1940	12	*d*
deutero-Mb	23 ± 3	*x*	1942	9.5	*d*
dibutenyl-Mb	30 ± 2	*x*	1941	12	*d*
diacetyl-Mb	170 ± 5	*x*	1950	12	*d*
diformyl-Mb			1954		*l*
cytochrome *o*	100	*m*	1963	9	*n*
hemoglobin	150	*o*	1951.5	8	*p*
Chironomous Hb	125	*q*	1962.4	8	*r*
leghemoglobin	190–230	*s*	1948.5	7	*p, t*
cytochrome a_3	365	*u*	1963.5	5.5[w]	*v*

[a] Values at 25 °C where available. Experimental errors are given for values determined in this study. [b] The P band (H band). The positions of the shoulders found to low energies in the spectra of various Mbs are not reported since ν_{CO} for these shoulders is dependent upon the curve-resolving technique used. [c] Yamada et al., 1975. [d] This study, measured at 20 °C, "pH" 7,in deuterated buffer. Does not affect P band parameters but may lower H band ν_{CO} by up to 3 cm^{-1}. [e] Conroy et al., 1978. [f] Iizuka et al., 1983. [g] Satterlee & Erman, 1984. [h] Ohlsson & Paul, 1983. [i] M. H. Gelb and S. G. Sligar, personnal communication. [j] O'Keeffe et al., 1978. [k] Caughey et al., 1982. [l] Tsubaki et al., 1980. [m] Webster & Hackett, 1966. [n] Choc et al., 1982. [o] Abraham & Taylor, 1975. [p] Caughey et al., 1978. [q] Brunori et al., 1971. [r] Wollmer et al., 1977. [s] Henderson & Appleby, 1972. [t] Fuchsman & Appleby, 1979. [u] Dutton et al., 1970. [v] Yoskihawa et al., 1977. [w] Lower values have been reported, obtained in other solvents and at other temperatures. $\Delta\nu_{1/2}$ = 5.5 cm^{-1} was determined at room temperature and in aqueous solution, essentially the same conditions as for other spectra in the table. Therefore, this value was chosen in spite of some instrumental broadening. [x] In 50 mM sodium phosphate–1 mM EDTA, pH 7.0, 25 °C.

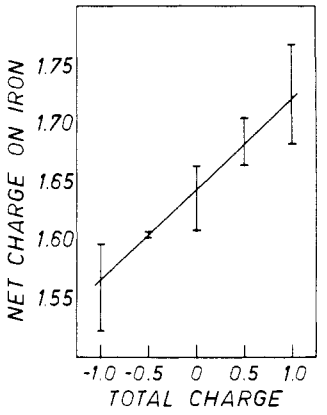


FIGURE 6: Calculated net positive charge upon iron vs. total charge upon $N_5C_{14}Fe{\cdot}CO$ complex.

creasing positive charge, corresponding to more positive E_m, ν_{CO} increases linearily. While the range of ν_{CO} covered by both experiment and theory is similar (30 cm^{-1}), the calculated values are numerically higher than those observed experimentally. Error bars in Figures 5, 6, and 8 indicate incomplete convergence between potential and wave function. Since we apply an m_l averaging procedure (Rosén et al., 1976), such discrepancies between potentials and wave functions are not defined when we separate σ and π contributions.

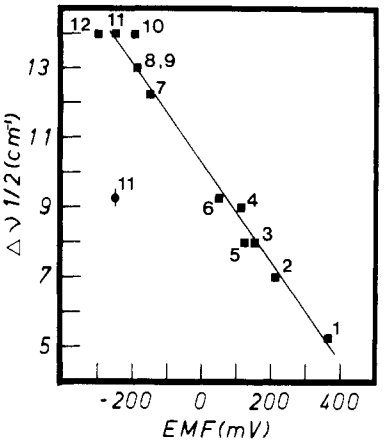


FIGURE 7: CO stretch width at half band maximum ($\Delta\nu_{1/2}$) vs. E_{m7} at pH 7.0 for native hemoproteins. Numbering as in Figure 4.

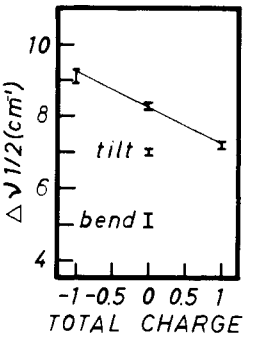


FIGURE 8: Calculated $\Delta\nu_{1/2}$ for the model $N_5C_{14}Fe{\cdot}CO$ vs. total charge upon complex.

In Figure 6 the net charge upon iron has been factored out from the total charge. Like metal surfaces, the porphyrin is a good delocalizing vehicle for electronic charge whether positive or negative (Paul et al., 1984). However, any "translation" of a change in molecular charge into a change in E_m, or vice versa, must proceed via observed shifts of ν_{CO}. A calculation of net charge balance between porphyrin and Fe must await calculations with all porphyrin atoms included.

Half-Bandwidth, $\Delta\nu_{1/2}$. The very close correlation between $\Delta\nu_{1/2}$ and E_{m7} for hemoproteins is shown in Figure 7. Cytochrome *o*, CTT, and CCP did not correlate in Figure 4 but fit nicely within the linear relationship in Figure 7. The only outlying $\Delta\nu_{1/2}$ value is that of the P band of HRPC at pH 7. However, at pH 11 $\Delta\nu_{1/2}$ becomes 14 cm^{-1}, which places HRPC·CO within the correlation. HRPC is stable at this pH, which is well above the p*K* of the hydrogen bond to CO, 8.8 (Barlow et al., 1976). The cause of this "width stealing" remains unknown to us. In the case of the 2,4-substituted hemoproteins, the $E_m - \Delta\nu_{1/2}$ correlation is less obvious; therefore, other effects interfere, possibly substituent bulkiness.

Theoretical calculations of the difference in CO stretch lifetime of the $N_5C_{14}Fe{\cdot}CO$ model gave the results in Figure 8. The trend toward increasing $\Delta\nu_{1/2}$ with increasing negative charge upon the model correctly mimics the situation experimentally seen for the hemoproteins, and the calculated absolute values of $\Delta\nu_{1/2}$ satisfyingly fit in with the observed midrange of $\Delta\nu_{1/2}$. The slope is of correct sign but less than that in the experimental situation. Calculations on a tilted conformation of CO with the linear Fe–C–O axis 45° from normal to heme revealed that $\Delta\nu_{1/2}$ was reduced by 1 cm^{-1} (Figure 7), an effect hardly perceptible at a resolution of 1 cm^{-1}. The most tilted position observed so far is 30°, in CCP (Poulous et al., 1980). The "bent" conformation assumed an Fe–C–O angle of 135°, which gives a more pronounced cal-

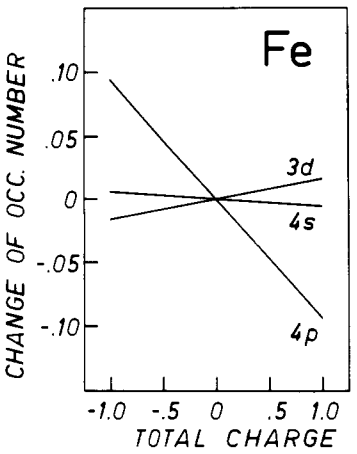


FIGURE 9: Calculated change of occupation number of the atomic orbitals on iron in the model $N_5C_{14}Fe{\cdot}CO$ vs. total charge upon complex.

culated decrease in $\Delta\nu_{1/2}$, about 3 cm^{-1} (Figure 8).

Finally, calculations show (Figure 9) that the Fe 3d and 4s orbital occupations remain nearly constant even at great changes of total charge upon the complex. Interestingly, most of the orbital occupation change takes place in 4p orbitals. It is the 4p orbitals within the porphyrin plane which, in combination with the 3d orbitals, are responsible for π back-bonding to the CO molecule.

Discussion

ν_{CO} responds uniformly to changes in E_{m7} by about 55 cm^{-1} V^{-1}, independently of whether heme or protein causes the alteration of E_m. This settles the paramount importance of the electronic structure of iron for the vibrational properties of heme-bound CO. It also points at the possibility of gauging protein-induced effects by measuring effects of 2,4-substitutions in heme, the latter being more accessible to theoretical considerations. From this linear response, some potentially informative deviations occur. Anomalously low ν_{CO} values, which appear only at low E_{m7}, are accounted for by hydrogen bonding between the protein and the oxygen atom in CO. Anomalously high values of ν_{CO} will be discussed in terms of distortions of the Fe–C–O bonds away from their conformation normal to heme. The half-bandwidth, $\Delta\nu_{1/2}$, of the natural hemoproteins is highly correlated to E_{m7} with a linear, negative slope. As long as the correlations hold for both vibrational parameters, there can be little H bonding or tilting/bending of CO. There is reason to suspect a steric effect on CO when ν_{CO} as well as $\Delta\nu_{1/2}$ are off their linear correlations to E_{m7}. There is probably a H bond to CO when ν_{CO} is less than predicted by the correlation and $\Delta\nu_{1/2}$ is normal. This may be of some diagnostic use.

Quantum mechanical calculations by the Xα method of a carbonylheme model confirm the covariation of ν_{CO} and $\Delta\nu_{1/2}$ with electron availability and show that these dependencies are due to a high density of metal–ligand electronic states around the Fermi level. Furthermore, charge accomodation between the so-called π electrons of the porphyrin and CO is mediated mainly via the Fe 4p orbitals with little involvement of the Fe 3d orbitals.

Hydrogen Bonding to CO. The shift of ν_{CO} from 1933 to 1903 cm^{-1} upon acidification of HRPC·CO has been attributed to hydrogen bonding of CO (Barlow et al., 1976) and so has an isotope effect on the ultraviolet–visible spectrum of HRPC·CO (Hayashi et al., 1976). Chemical modification of HRPC by the histidine-specific reagent *p*-chlorobenzoyl chloride completely prevented the 1903-cm^{-1} band in HRPC·CO, suggesting histidine as a proton donor (Schonbaum et al., 1979). Hydrogen bonding to CO in HRP·CO (Smith et al., 1983) and CCP·CO (Satterlee & Erman, 1984) was recently confirmed by ^1H–^2H exchanges. A rule of thumb, derived from Figure 4, may be that ν_{CO} <1920 cm^{-1} for hemoprotein–CO indicates hydrogen bonding to CO. The presence of a hydrogen bond between heme-bound dioxygen and protein is well established for crystalline $Mb{\cdot}O_2$ (Phillips & Schoenborn, 1981) and $Hb{\cdot}O_2$ (Shaanan, 1982) and for these proteins in solution (Kitagawa et al., 1982).

Hydrogen bonding in CO as an explanation of lowering of CO bond order was not used by other authors discussing the effects of protein or model structure upon heme-bound CO (Caughey et al., 1979; Collman et al., 1976; Momenteau & Lavalette, 1982; Momenteau et al., 1983). However, at increasing electron density on the cluster model of heme, the CO carbon atom carries a constant, negative charge whereas the oxygen atom becomes increasingly more negative and hence more apt to accept a hydrogen bond (Paul & Rosén, 1984). This is in line with the experimental observations that peroxidases, which have low E_m values, form Fe–CO···H–X bonds whereas the more positive Hb and Mb do not.

Covariance of $\Delta\nu_{1/2}$ with E_{m7}. The plot of $\Delta\nu_{1/2}$ for the P frequencies of native proteins vs. E_{m7} (Figure 7) shows that the two probes of hemoprotein active centers are related. Since the correlation is good, $\Delta\nu_{1/2}$ must be thought of as an intrinsic property of hemoprotein electronic structure. The observed difference of $\Delta\nu_{1/2}$ over the range of proteins studies is about 9.5 cm^{-1}, $\Delta\nu_{1/2}$ increasing with decreasing E_{m7}. The calculated dependence of $\Delta\nu_{1/2}$ upon total complex charge for the $N_5C_{14}Fe{\cdot}CO$ model presented in Figure 8 also indicates an increase of $\Delta\nu_{1/2}$ with increasing electron availability. This effect arises from stronger back-bonding between Fe and CO as E_m is lowered and negative charge is introduced into the carbonylheme. Since charge can more easily flow between heme and CO in the more strongly bonded complexes, δ_n, and therefore $\Delta\nu_{1/2}$, reflects the bonding strength between Fe and CO. Hydrogen bonding of CO will increase $\Delta\nu_{1/2}$ (Table I).

Whereas ν_{CO} can be resonably well evaluated from a limited model like $N_5C_{14}Fe{\cdot}CO$, δn, the oscillating charge, is much more sensitive to model size. However, in spite of the fact that the applied algebraic formula is an approximation, the strong correlation of $\Delta\nu_{1/2}$ and ν_{CO} with E_{m7} favors a mechanism based upon carbonylheme electron distribution.

Inspection of the table reveals that $\Delta\nu_{1/2}$ somewhat responds to the steric size of heme 2,4-substituents. For both HRPC·CO and Mb·CO, diacetyl- and mesoheme substitutions increase $\Delta\nu_{1/2}$, while values of the deuterioheme-substituted proteins are similar to those of native proteins. The uniform response to acetyl and ethyl substitutions excludes a pure charge effect. A reasonable explanation is that substitution by hemes with bulky 2,4-substituents perturbs the protein around the ligand binding pocket. A crystallographic study has shown that perturbation of the protein environment is minimal in deuterioheme hemoglobin and more extensive in mesoheme hemoglobin (Seybert & Moffat, 1976, 1977). It is not clear from our data whether such a disturbing effect of protein conformation translates itself into increased $\Delta\nu_{1/2}$ by electronic effects through the heme or by a direct effect upon the CO vibrator.

As just stated, $\Delta\nu_{1/2}$ of native protohemoproteins is a function of E_{m7} (Figures 7 and 8), which again is settled by the protein moiety. Stellwagen (1978) observed a linear correlation between E_m and the protein–heme interface area. Such an influence can hardly operate exclusively via the proximal histidine but requires a direct protein–porphyrin

interaction. A corollary is that information on ligand binding geometry and direct protein steric effects upon ligand cannot be ascertained from a $\Delta\nu_{1/2}$ value, which obeys the linear correlation to E_m. The very small $\Delta\nu_{1/2}$ for carbonyl-cytochrome *c* oxidase does not necessarily mean that the ligand binding site is highly ordered as has been stated (Yoshikawa et al., 1977; Choc et al., 1982; Caughey et al., 1976).

Covariance of ν_{CO} and E_{m7}. Plots of ν_{CO} vs. E_{m7} give a seemingly scattered picture (Figure 4). Some results are, however, most likely due to factors in addition to heme electronic structure. We will first discuss such values.

We have previously argued that values of $\nu_{CO} < 1920$ cm^{-1} stem from hydrogen-bonded CO. In Figure 4, a cluster of six values of this kind is easily identified. Next, the ν_{CO} values of CTT Hb (6), CCP (9), cytochrome *o* (4), and cytochrome P-450 (12) appear high. While we have no definite explanation for these four deviations, we will point at some circumstances that seem to sequester them from other hemoproteins in this study.

The excessively high value of ν_{CO} for *Chironomous* hemoglobin must be connected to a reduced π back-bonding, i.e., a higher C–O force constant and a lower Fe–C force constant (Fuchsman & Appleby, 1979; J. Paul, M. L. Smith, and K. G. Paul, unpublished results). Steigemann & Weber (1979) found by X-ray crystallography at 1.4-Å resolution that CO in CTT III Hb·CO suffered an unfavorable bond geometry due to steric hindrance by the globin moiety, expressing itself as a very long Fe–CO distance, 2.4 ± 0.1 Å (1.8 Å for Fe–O in CTT Hb·O_2). Part of the strain was relieved by moving the carbon atom 0.3 Å off the normal to heme through the iron atom. Thus, although the Fe–C bond length seems startling, X-ray data and the off-position of our ν_{CO}/E_m value fit together. We regard a reduced π back-bonding because of steric hindrance to be the cause of the excessively high ν_{CO} of CTT Hb·CO.

A second protein for which ν_{CO} and E_{m7} do not correlate in Figure 4 is cytochrome *c* peroxidase. At pH values above neutrality the P band appears at about 1949 cm^{-1} (Iizuka et al., 1983; Satterlee & Erman, 1984), which is 8 cm^{-1} more than that of carbonyllactoperoxidase (1941 cm^{-1}), both with similar E_{m7} values (ca. −0.19 V). The crystallographic structure of the Fe(III) protein shows the iron liganding water and the proximal histidine lying about 30° from the axis normal to heme with the iron atom displaced about 0.4 Å toward the proximal histidine (Poulos et al., 1980; Skoglund, 1979). The removal of Fe from CCP does not alter the position of the distal histidine, as judged from X-ray crystallography (Skoglund, 1979). In Hb·CO and Mb·CO, the iron atom lies closer to the mean porphyrin plane, and the proximal histidine is much closer to heme than in CCP (Baldwin, 1980; Hanson & Schoenborn, 1981). This perturbation of the iron–porphyrin geometry may lengthen the Fe–C bond and lower this bond order while increasing the C–O bond order and ν_{CO}.

The three-dimensional structure of cytochrome *o* is not known. The IR spectra of Hb·O_2 and Mb·O_2 are complicated with up to six bands in the O–O stretch region between 1155 and 1095 cm^{-1} (Caughey et al., 1982). These complicated spectra arise from Fermi coupling between the Fe–O and O–O vibrations (Alben et al., 1978), possibly also from coupling through a hydrogen bond to vibrations within the distal histidine. Cytochrome *o* exhibits only one O–O stretch in the infrared region (Choc et al., 1982), which means a ligand environment different from Hb and Mb. This could possibly reflect in the abnormally high ν_{CO} of the carbonyl complex.

Cytochrome P-450$_{cam}$–CO with camphor present exhibits a single band at 1940 cm^{-1} ($\Delta\nu_{1/2}$ = 12.5 cm^{-1}). In the absence of camphor, a second band arises at 1962 cm^{-1} ($\Delta\nu_{1/2}$ = 12–16 cm^{-1}) in a similar position as the A band of HRPC·CO (ν_{CO} = 1962 cm^{-1}, $\Delta\nu_{1/2}$ = 10–12 cm^{-1}), the denatured form of P-450 known as P-420 (ν_{CO} = 1965 cm^{-1}, $\Delta\nu_{1/2}$ = 25 cm^{-1}), and the denatured forms of Hb·CO (ν_{CO} = 1966 cm^{-1}, $\Delta\nu_{1/2}$ = 20 cm^{-1}) (Peterson, 1979). There is a possibility that P-450 in the absence of camphor spontaneously releases heme (Hockin & Paine, 1983) and this gives rise to an A-type band at 1962 cm^{-1}. The loss of heme from P-450 is considered a major problem of the isolation (Imai et al., 1980; Haugen & Coon, 1976; Boström et al., 1982). Also, P-450 is unique, having a proximal sulfur ligand to iron, with unknown IR implications.

The remaining data in Figure 4 fairly well correlate ν_{CO} to E_{m7} in a linear fashion. Hemoproteins with ν_{CO} below 1920 cm^{-1} suffer hydrogen bonding to CO, and hemoproteins with ν_{CO} above the correlation line in Figure 4 place steric restraints upon the CO vibrator. We have checked this hypothesis by substituting two monomeric hemoproteins, HRPC and sperm whale Mb, with various artificial hemes. Substitution with artificial hemes alters E_{m7} of HRP (Yamada et al., 1975) and ν_{CO} of Hb and Mb (Wallace et al., 1982; McCoy & Caughey, 1971). The data (Figure 3, Table I) show that substitution of protoheme with artificial hemes in both HRPC and Mb changes E_{m7} and ν_{CO} in an orderly fashion. Not only that but the dependence of ν_{CO} upon electron density at iron, as expressed by E_m [Zerner et al. (1966) and references cited therein] follows a trend very close to that expressed by well-behaving native proteins.

The trend of decreasing ν_{CO} with decreasing E_m is nicely confirmed by theoretical calculations shown for the N_5C_{14}-Fe·CO model (Figure 5). Our calculated values, while being of higher energies, show that ν_{CO} is responsive to changes of electron density upon the heme. These calculations confirm that heme electron density must be considered as playing the basic role of determining ν_{CO}.

Substitution of Mb with 2,4-diacetylheme raises E_{m7} from 0.043 V in the native protein to 0.170 V, close to 0.150 V observed for human Hb (Antonini et al., 1964). The observed ν_{CO} for 2,4-diacetylheme (Mb·CO is 1950 cm^{-1}, also close to the 1951.5-cm^{-1} ν_{CO} of human Hb·CO. According to these data, the cause of a lower ν_{CO} in Mb·CO compared to Hb·CO is increased electron density at iron in Mb. It is not necessary to invoke steric restraint to account for the difference in ν_{CO} between Hb·CO and Mb·CO. This conclusion is similar to that reached by Mims et al. (1983), who have shown that the energetics of methylisocyanide–Hb and linear and bent ethyl isocyanide–Hb complexes are equivalent.

Electronic Distribution within Hemoproteins. Theoretical and experimental results agree as regards sign and magnitude of ν_{CO} and $\Delta\nu_{1/2}$, which justifies the approach taken in the calculations. The connections between ν_{CO}, $\Delta\nu_{1/2}$, and E_{m7} can be described as follows. When CO has a high density of states, with strong influence on ν_{CO} (e.g. CO $2\pi^*$) around the Fermi level (E_F), ν_{CO} will be sensitive to a small shift of E_F, i.e., to E_m. Increasing the filling of a broadened state around E_F will enhance the finite number of electrons oscillating to and from CO (δn) when the molecule is vibrationally excited. The square of this number is approximately proportional to $\Delta\nu_{1/2}$ according to a theory for CO adsorbed onto metal surfaces (Persson & Persson, 1980; Ueba, 1982). Also, within the order of magnitude of a few hundredths of an electron, δn in very sensitive to even small changes in DOS around E_F. Extended

and even more precise calculations therefore have to be based on the complete heme molecule.

For heme·CO, iron 3d states are well represented in a t_{2g}^6 e_g^0 configuration caused by a strong ligand field of approximate O_h symmetry (Paul et al., 1983). The insensitivity of the occupation of 3d states to changes in E_m can qualitatively be understood by the low 3d DOS at the highest occupied orbital (Figure 9). Within our set of basis functions, charge accomodation between so-called π electrons on the porphyrin and the axial ligand is instead mediated via Fe 4p electrons. The separation of 3d states will, however, respond to changes in the ligand field formed by the pyrrole nitrogens. We regard the Fe–C bond as predominantly a CO 5σ–Fe $3d\sigma$ charge transfer plus a Fe $3d\pi$–CO 2π back-bonding with nonnegligible C 2p–Fe 4p overlap, as documented in previously calculated population matrices (Case et al., 1979; Herman et al., 1980; Loew & Kirchner, 1978). We believe the 4p contribution in the porphyrin plane to be correlated with E_m. Walters & Spiro (1982) found in a resonance Raman study a direct coupling of Fe–O_2 to the porphyrin π–π^* transition, which they explained by competition between π^* orbitals of porphyrin and O_2 for Fe 3d electrons. This is in line with our results, but we regard 4p electrons as the transfer medium. The charge oscillations between CO and heme, when CO is vibrationally excited, operates via these electrons as well. Finally, we note that our separation of Fe electronic orbitals into localized 3d states and delocalized p states is paralleled by the electronic configuration in transition metals. Here, 3d states show only little dispersion whereas sp electrons are regarded as free-electron-like.

Protein Pocket. Clearly, $\Delta\nu_{1/2}$ covaries, in a consistent manner, with changes in electron availability at the iron atom, either the changes are brought about by equatorial substitutions in the heme group, by hydrogen bonding of CO, or by protein-induced effects, registered as E_{m7}. The two first mechanisms have been discussed above. Does the protein moiety exert its effects on $\Delta\nu_{1/2}$ by allowing more randomness to liganded CO (Caughey, 1971; Caughey et al., 1978) or via E_{m7} and electron distribution in heme?

Stellwagen (1978) observed an inverse linear correlation between E_{m7} and the extent of exposure of the heme group to water as computed from X-ray data. His selection did not include any hemoproteins with $E_{m7} < 0$; most of them were of the cytochrome *b* or *c* type, which do not give a CO complex. Nevertheless his study is of interest since it points out the possible importance of the fourth component (water) in the balanced interplay in a hemoprotein. Crystallographic analyses of cytochrome c_3 (Pierrot et al., 1982) revealed highly exposed hemes, which together with a very low E_m supports Stellwagen's conclusion. So does also the observation (Ohlsson et al., 1984) that the rate constants and activation energies for the formation of compound I of HRP C, A, and lactoperoxidase with dihydrogen and various alkyl hydrogen peroxides suffered increased steric hindrance as E_{m7} increased. From Stellwagen's correlation and Figures 7 and 8, it follows that $\Delta\nu_{1/2}$ should increase as the exposure of heme to the surrounding solvent water increases. This is well compatible with the concept of $\Delta\nu_{1/2}$ depending upon the space allowed to liganded CO. However, it is hard to see how H bonding of CO could increase space and randomness. We would like to offer a chemical explanation for Stellwagen's correlation and show how it can be applied to hemoproteins with a low E_{m7}.

The electronic distribution within the porphyrin moiety is highly responsive to the local environment. Buried in a hydrophobic pocket, the porphyrin receives little electron density from the hydrocarbon-like environment. When porphyrin is allowed the freedom of a water environment, it functions as an electron sink, receiving electron density from water, which acts as a Lewis base. For hemoproteins with a low E_{m7}, like peroxidases, another donor seems to be at hand. In CCP, which has a deep pocket but still a low E_{m7}, a buried, a distal guanidinium group in contact with pyrrole IV (Skoglund, 1979; Poulos et al., 1980) takes the donor function of water. Such a buried guanidinium group is also found in catalase, where the side chain of Arg-353 is in contact with pyrrole I (Murthy et al., 1981). The distal sequences of plant peroxidases contain a conserved arginyl residue (Welinder, 1976). Our contention is that an uncharged, buried guanidinium group is the chemical reason why peroxidases have $E_{m7} < 0$. Interestingly, ^{13}C NMR spectra of carbonyl hemoproteins correlate with ν_{CO} (Satterlee, 1983).

This construction unites the two apparently different mechanisms for the protein effect on $\Delta\nu_{1/2}$. The very high correlation between E_{m7} and $\Delta\nu_{1/2}$ ties the two parameters directly and closely together. Increased space for liganded CO will increase $\Delta\nu_{1/2}$ but also make heme more exposed to water. This again will lower E_m and, consequently, increase $\Delta\nu_{1/2}$. Thus, the space effect operates via the electron distribution and E_m. For peroxidases, an additional donor to heme operates, taking the function of water.

Acknowledgments

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Supplementary Material Available

Figure of infrared spectra of the carbonyl hemoproteins lactoperoxidase, HRPA, legHb, and CCP and figure of the carbonyl hemoprotein HRPC substituted with deuteroheme, 2,4-diacetyldeuteroheme, and mesoheme (2 pages). Ordering information is given on any current masthead page.

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Thermal Coefficient of the Frictional Resistance to Rotation in Simple Fluorophores Determined by Fluorescence Polarization†

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ABSTRACT: Experimental data are presented by employing organic fluorophores, tryptophan and tyrosine among them, that substantiate a logarithmic expansion of the viscosity as a function of temperature for the determination of the thermal coefficient of the viscosity by measurements of the polarization of the emitted fluorescence. The values obtained agree within experimental uncertainties with those determined by flow viscometry, and the agreement extends to a range of glycerol–water mixtures for which the thermal coefficient of the viscosity is an irregular function of the glycerol content. Prodan, a fluorophore that forms strong bonds with the hydroxylic solvents employed, yields values similar to those obtained with perylene, which interacts with solvent by weak dispersion forces alone.

A number of studies have shown that the polarization of the fluorescence of tyrosine and tryptophan in proteins is determined by the overall rotation of the protein molecules as well as by rotations of local character in which the individual residues undergo motions independent of those of the whole particle. Such partial motions are revealed by Perrin plots (Perrin, 1926) with limiting polarization values smaller than those that correspond to a truly immobile fluorophore (Weber, 1952) or by an initial slope that corresponds to a kinetic rotational volume much smaller than that of the whole protein (Wahl & Weber, 1967; Lakowicz & Weber, 1980). Both characteristics are equally unequivocal in those cases in which the emission is due to a single excitable fluorescent residue, but the decrease in apparent limiting anisotropy may contain

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