Association of Imidazoles and L-Histidine with the Pentacyanoiron(II) and -(III) Moieties

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Abstract: The Fe(II) and Fe(III) complexes having the general formula $(CN)_5Fe(imidazole)^{n-}$, $n = 3$ or 2, have been characterized for a series of substituted imidazoles including histidine. Association constants for the N-3 bound imidazole moiety are 7.9×10^5 (imidazole) and 4.1×10^3 (4,5-dimethylimidazole), implicating a strain energy of about 3.1 kcal/mol for methylation adjacent to the iron center. The low-spin $(CN)_5Fe(imidazole)^{3-}$ complex is found to be 5.5 kcal/mol more stable than high-spin $(H_2O)_5Fe(imidazole)^{2+}$. The Fe(III) complexes exhibit a ligand-to-metal charge-transfer band near 500 nm that is absent in the pyridine complexes. The LMCT band is sensitive to the inductive nature and total number of ring substituents. This band is energetically similar in the Ru(III) ammine analogues; inductive effects obey the order C-4 > C-5 > C-2. The pK_a of the pyrrole hydrogen in the Fe(III) imidazole complex is 10.5 ± 0.1, similar to the ferrimyoglobin complex. A quasi-reversible cyclic voltammetric wave is observed at Pt with $E' = 0.365$ V and peak separation of 141 ± 5 mV in 0.10 M $NaClO_4$ at 25 °C for the Fe(III)/Fe(II) couple.

Introduction

Imidazole and its derivatives are of considerable interest in their role of ligands for transition metal ions because of the biological binding of histidine to metal ion centers in various metalloenzymes and heme proteins. Ligation of the metal in the active site in so many systems, particularly with iron in the cytochromes and hemoglobin, by imidazole implies that a more-than-chance evolutionary selection process ultimately has incorporated this unit as having special, needed chemical features. What are these features? Since the role of these biological systems is to function either by cyclic changes in oxidation state, alterations in the spin state, or conformation changes coupled to changes in electron density on the metal center, it is most likely that imidazole serves to assist by inductive effects or perhaps redox reactivity of its own. Traylor has suggested that the large π donor capacity of imidazole interacts through the t_{2g} system to stabilize the Fe(II) state of hemoglobin and synthetic O_2 carriers. This function is equivalent to the assisted charge dispersal which stabilizes $(Fe(III)\text{-}O_2^-$ and may cause induced confirmation changes in the hemoglobin protein structure.[1] Wang has postulated a genuine redox role in which the Fe(II) center and imidazole undergo a concerted $2e^-$ oxidation to form a radical precursor in steps leading to high-energy phosphorylated compounds.[2] However, the imidazole radicals themselves have yet to be characterized in the systems containing iron in any oxidation state. The second member of the iron triad reveals a new feature in the reactivity of imidazole with metal centers. Ruthenium(II) has shown the ability to coordinate to imidazole via the normal N-3, pyridine nitrogen or to the C-2 position of the ring as an imidazolium ylide.[3,4] Histidine may also adopt N-3 or C-2 coordination in ruthenium(II) ammines.[5] With the exception of Ru(II) the only observed bonding mode of neutral imidazole to other transition metal ions has been to the sp^2 pyridine nitrogen. However, a linkage isomerism between N-3 and C-2 of histidine has potential biological importance as a control feature. A rearrangement within a metalloprotein from N-3 to C-2 coordination of a histidine unit might produce the necessary conformation change or a change in the midpoint reduction potential of a metalloprotein.[3] The interconversion between N-3 and C-2 binding could also provide a trigger for biological control, dependent on external conditions. For example, imidazolepentaammineruthenium(II) isomerism from N-3 to C-2 is acid catalyzed.[3]

We wish to report our initial observations on imidazolepentacyanoiron(II) and -(III) and a related series of complexes, including histidine. Our studies explore the ability of imidazoles to effect the following interrelated aspects of the chemistry of the $(CN)_5Fe^{3-}$ and $(CN)_5Fe^{2-}$ moieties: (1) stabilization of the Fe(II) and Fe(III) oxidation state as a π acceptor and a π donor; (2) possible formation of carbon-bound analogues of the imidazole complexes of the ruthenium(II) ammines; (3) the kinetics of oxidation of the Fe(II) state by H_2O_2; (4) the catalase activity of the Fe(III) state; (5) possible evidence for Fe(IV) or the isoelectronic Fe(III)-imidazole radical entity. This report presents the data concerning the interaction of imidazoles with the pentacyanoiron(II) and -(III) units. The reactivity of imidazolepentacyanoiron(II) and -(III) with H_2O_2 is discussed in a future paper.

Experimental Section

Electronic Spectra. The $Na_3Fe(CN)_5NH_3 \cdot 3H_2O$ was prepared by the reduction of $Na_2Fe(CN)_5NO$ with NH_3 in the presence of NaOH followed by recrystallization from concentrated NH_3. Complexes were prepared in solution by replacement of solvent water from the aquated $Fe(CN)_5NH_3^{3-}$ by other desired ligands. Extinction coefficients were measured relative to the literature value of the pyrazine complex, $(CN)_5Fe(pyr)^{3-}$.[6] Uv-visible spectra were obtained using a Cary 15 spectrophotometer calibrated using a holmium oxide glass filter. These results are summarized in Table I.

NMR Spectra. Spectra were obtained using a Varian T-60 NMR or a Perkin-Elmer P-32 NMR; service spectra were obtained using a Varian XL-100 NMR.[7] *tert*-Butyl alcohol was used as an internal reference. Changes in pD were achieved by addition of trifluoroacetic acid to samples of weighed composition in D_2O. Spectra from the XL-100 were obtained on samples prepared under N_2, prescrubbed through Cr(II) solution. The NMR tubes were also flushed, capped by rubber septa, and filled using syringe techniques.

Anion Exchange. The $(CN)_5Fe(imz)^{3-}$ complex was isolated by charging a Dowex AG-4X resin in the Cl^- form. Resins were exhaustively cleaned with HCl, NaOH, H_2O_2, acetone, and ethanol before final conversion to either the Cl^- or SO_4^{2-} form by HCl or H_2SO_4 solutions. The $(CN)_5Fe(imz)^{3-}$ was eluted by 1.00 M NaCl in an inert atmosphere ion exchange column containing N_2 preflushed resin. The $(CN)_5Fe(imz)^{2-}$ was prepared by H_2O_2 oxidation of $(CN)_5Fe(imz)^{-3}$. The Fe(III) state was separated from H_2O_2 in air-exposed resin columns and eluted by 4.0 M NaCl without apparent decomposition in the resin phase for $(CN)_5Fe(imz)^{2-}$. The Fe(III) 4-methylimidazole complex underwent decomposition under similar conditions, forming Fe(II) and Fe(III) mixtures by an undetermined route in both the Cl^- or SO_4^{2-} columns.

Cyclic Voltammetry. A potentiostat was assembled using a Wavetec 133 function generator and a conventional operational amplifier arrangement. Triangular-wave cyclic voltammetry was carried out under N_2 at a Pt minibar working electrode separated by a coarse glass frit

from a Pt mesh auxilliary electrode. Potential measurements were made vs a saturated calomel electrode. Voltage measurements were made using an Orion 601 Digital pH meter on the millivolt scale. Current-voltage curves were displayed by a Houston 2000 X-Y recorder. Reliable behavior for the equipment was shown by standardization with the $Ru(NH_3)_6^{3+,2+}$ couple in 0.10 M phosphate buffer. A reversible wave with a peak-to-peak separation of 78 mV was obtained at 0.08V vs. the hydrogen electrode. The solvent blank and Sigma Grade III imidazole gave no electrochemical wave in the same region as Figure 3 nor was the behavior of $(CN)_5FeH_2O^{3-}$ the same as the complex in 0.10 M NaCl or 0.10 M phosphate buffer.

Imidazole Compounds. Imidazole was used as supplied by Matheson Coleman and Bell. The 4-methylimidazole was purchased from K & K. The 2,4-dimethylimidazole was obtained from Gallard-Schlesinger and purified by repetitive sublimation. The 4,5-dimethylimidazole was synthesized by a literature procedure[8] and purified by vacuum distillation. The product was stored under N_2 in a desiccator. The 4,5-dimethylimidazole undergoes rapid air oxidation in solution, particularly CH_2Cl_2, as well as a slower oxidation of the solid state. The imidazole-*2-d* was synthesized by base-catalyzed exchange of the C-2 position in D_2O. The imidazole-*2-d* was recovered and analyzed by NMR showing the total loss of the C-2 resonance at δ 7.79.

Oxidation of $(CN)_5Fe(imz)^{3-}$. Conversion of the Fe(II) complex to Fe(III) was achieved by oxidation with known amounts of H_2O_2. Attempts to measure an E° for the III/II couple yielded non-Nernst behavior in agreement with later studies by cyclic voltammetry. The indicator electrode was a Pt foil electrode while the reference was a standard Ag/AgCl electrode; the cell had a glass frit junction. Electrochemical and pH data were recorded at 25.0 °C on an Orion Digital 601 pH meter calibrated at the shortening potential internal standard or commercial buffers. The same product of the H_2O_2 oxidation was also produced by oxidation with I_2, OCl^-, $Fe(CN)_6^{-3}$, O_2, or electrochemically at a graphite rod electrode.

$Zn(CN)_5Fe(imz)\cdot 2.5H_2O$. From solutions containing $(CN)_5Fe(imz)^{2-}$ a solid could be obtained by precipitation with $ZnCl_2$ solution. Its composition analyzed best for a 2.5-hydrate. Anal. Calcd: C, 26.4; N, 26.9; H, 2.47. Found:[9] C, 27.1; N, 27.3; H, 2.49.

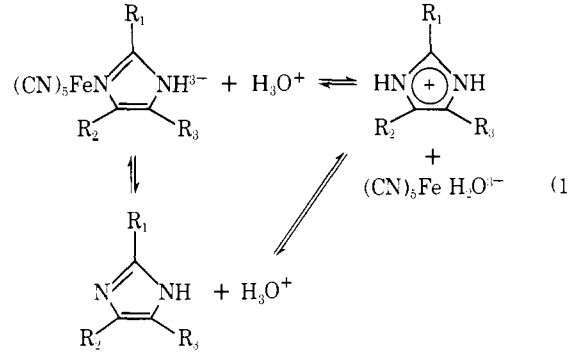
Equilibrium Constant Determinations. Known amounts of 2-methylpyrazine were added to temperature equilibrated flasks containing weighed amounts of imidazole at equilibrium with $(CN)_5Fe(imz)^{3-}$. After allowing equilibrium to be established between Ia and IIa based on the exchange time of Ia, spectra were obtained and the amount of IIa was determined by its absorption at a 450 nm. The amount of Ia was calculated from the total Fe(II) present. Mass balance in total Fe(II) was then checked by addition of a large molar excess of 2-methylpyrazine to force complete formation of IIa. The amount of Fe(II) found was equal to the initial amount. A similar procedure was used to evaluate the formation constant of If.

The rate of substitution of imidazole on $(CN)_5FeH_2O^{2-}$ was studied by mixing imidazole with a solution of $(CN)_5FeH_2O^{3-}$ incubated for 3 h with a 100-fold excess of H_2O_2. The reaction was complete in about 6.0 s at 0.33 M imidazole.

Results

Imidazole Complexes of $(CN)_5Fe^{3-}$. Although $(CN)_5FeH_2O^{3-}$ has been known to form adducts with nitrogen bases for over 70 years, no imidazole complex has been reported. Malik et al. have reported that $(CN)_5FeH_2O^{3-}$ fails to form a 1:1 complex with histidine without prior loss of CN^-.[10]

We have examined the substituted imidazoles in association with $(CN)_5Fe^{3-}$ by NMR and spectrophotometric techniques for the series of ions in Table I for compound I. Addition of $Na_3Fe(CN)_5NH_3\cdot 3H_2O$ to imidazole buffer under N_2 produces a solution having a spectrum with a single maximum at 382 nm. The spectrum of Ia remains unchanged after separation from the free ligand by anion exchange. Samples of Ia-g prepared in D_2O and acidified with trifluoroacetic acid to convenient values of pD reveal distinct resonance for protons of the bound and free imidazoles. Distinct resonances for ring protons and methyl groups for Ia-g imply that the exchange time is slow with respect to the NMR time scale. The methine and methylene hydrogen resonance of L-histidine are also altered by association of L-histidine with $Fe(CN)_5^{3-}$. The free ligand imidazole remains in rapid exchange with the corresponding imidazolium ion. Over the pD range of 11.9 to 4.9 only the N-bound isomer was observed for Ia. At higher

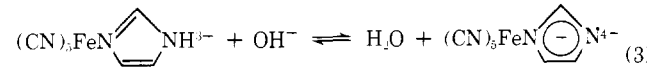


(1)

acidity, H_3O^+ competes with $(CN)_5Fe^{3-}$ for the lone pair of the imidazole pyridine nitrogen as shown by eq 1. This equation was studied as a function of pD for Ia, Ib, If, and Ig. At the high molarities required for the NMR experiments the dissociation as shown in eq 1 is further shifted by the coupled dimer formation equilibrium of eq 2 reported by Enschwiller.[11] A substantial fraction of the total Fe(II) present is complexed by an imidazole species for pD > 4.9 for Ia and pD > 7.0 for If.

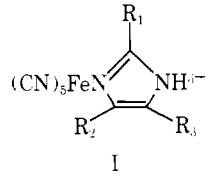
$$2(CN)_5FeH_2O^{3-} \rightleftharpoons 2H_2O + Fe_2(CN)_{10}^{6-} \qquad (2)$$

The C-2 resonance of Ia exhibits a small shift as a function of pH to higher values with increasing base strength. Equation



(3)

3 is suggested by the pH dependence on the C-2 resonance chemical shift. An attempt to titrate ion-exchanged Ia with NaOH failed to give a distinguishable pK_a below 10.5. The titration of Ia is accompanied by a very small increase in absorbance in the long-wavelength region of the absorbance band near 450 nm. Table II provides the data of the NMR spectra for Ia as a function of pD. Assignment of the chemical shifts has been made on the basis of the spectra of imidazolepentacyanoiron(II)-*2-d* (Ib) and 4-methylimidazolepentacyanoiron(II) (Id). The NMR data for complexes Ia-g are given in Table III at a pD of 10.



I

Table I. Spectral Data for $(CN)_5FeL^{3-}$ Complexes[a]

I	R_1	R_2	R_3	λ_{max}	$\epsilon \times 10^{-2}$
a	H	H	H	382	4.63
b	D	H	H	382	...
c	CH_3	H	H	387	4.94
d	H	H	CH_3	383	4.29
e	CH_3	H	CH_3	383	5.09
f	H	CH_3	CH_3	389	5.60
g	H	H	$^-O_2CCH(CH_2)$-NH_3^+	381	4.36

[a] Substituted imidazoles were purified by sublimation, crystallization, or chromatography and analyzed by NMR and mass spectrometry. Commercial histidine was used. T = 25 °C, μ = 0.10 NaCl.

Table II. NMR Data for Ia

	δ for bound ligand[a]		
pD	C-2	C-4	C-5
11.90	7.81	7.23	6.98
10.46	7.79	7.21	6.99
8.70	7.83	7.18	6.99
7.56	7.74	7.20	7.00
5.93	7.69	7.20	6.96

[a] *tert*-Butyl alcohol internal reerence; XL-100 NMR δ ± 0.03 ppm, T = 25 °C.

Table III.[a] Chemical Shifts for Substituted Imidazolepentacyanoiron(II) Complexes and Substituted Imidazoles at pD ≃ 10

	δ for bound ligand resonance			δ for free ligand resonance		
I	C-2 (R_1)	C-4 (R_2)	C-5 (R_3)	C-2	C-4	C-5
a	7.80	7.20	6.98	7.79	7.15	7.15
b		7.30	7.00		7.19	7.19[b]
c	2.90	7.22	6.81	2.36	6.97	6.97
d	7.70	6.90	2.16	7.62	6.80	2.19
e	(3.22)	(6.98)	(2.10)	2.27	6.59	2.10
f	7.73	2.40	2.07	7.57	2.08	2.08
g	7.78	7.04	3.04 (3.70)	7.73	7.04	3.19 (3.97)[c]

[a] T = 25 °C, $\mu \sim 4$, XL-100 NMR, *tert*-butyl alcohol internal reference. [b] T-60 NMR, 37 °C. [c] Perkin-Elmer P-32 NMR, 34 °C, pD 9.4. C-5 is the methylene resonance; those in parentheses are the methine resonance.

No exchange of the C-2D to C-2H occurred at pD 11 after 72 h at 25 °C for either bound or free ligand. These observations imply an absence of any C-2 bound isomer even at kinetically active levels which provide an exchange mechanism for C-2D. Ib displayed the same dissociation behavior as Ia in competition with H_3O^+.

When a methyl group appears in an adjacent position to the $Fe(CN)_5^{3-}$ unit, a less favorable equilibrium is established by reaction 1. A smaller fraction of Fe(II) is found as Ic compared to Ia and a slight broadening is observed in the methyl resonance of the complex. The C-2 methyl group in Ie is significantly broadened; the shift of the C-5 methyl group is hidden by the free ligand resonance. That all of the $(CN)_5Fe^{3-}$ is complexed in Ic, Ie, or If in the presence of excess ligand is confirmed in the visible spectra by the similar maxima and extinction coefficients comparable to Ia and the oxidation products of Ia–g, which retain the imidazole unit.

4,5-Dimethylimidazole under buffered conditions at pH 5.5 associates with Ru(II) as the C-2 bound isomer.[3] In association with $(CN)_5Fe^{3-}$, it shows two singlets for the C-4 and C-5 methyl groups and a C-2H resonance at δ 7.73 (If). No resonance attributable to a C-2 isomer was observed in the presence of excess free ligand from pD 11.0 to 7.0. At pD 7.0 dissociation occurs. Loss of the bound ligand resonances at pD 7.0 for If implies a greater stability for Ia of about 10^2.

2-Methylpyrazine Complex of $(CN)_5Fe^{3-}$. Addition of excess 2-methylpyrazine to Ia–f converts the Fe(II) present into 2-methylpyrazinepentacyanoiron(II) ion (IIa). The compound IIa exhibits a reversible protonation in two steps, observable as shifts in the CT band. One proton titrates the remote pyrazine nitrogen and the second associates with the cyanide ligands (cf. ref 5 and 6). Spectral data are listed in Table IV. IIa's NMR spectrum shows distinct methyl singlet resonances at δ 2.44 (bound) and 2.53 (free ligand) and ring proton

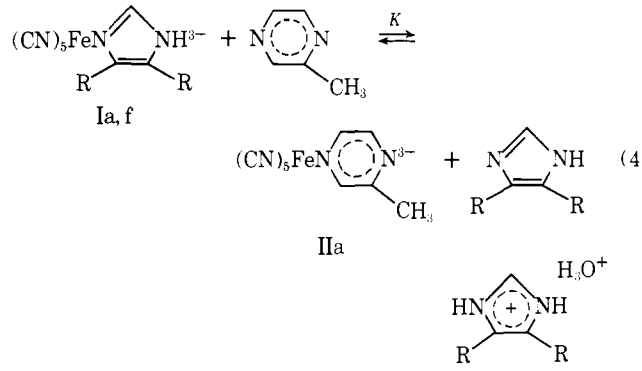
Table IV. Uv-Visible Spectra of IIa[a]

Species	$[H_3O^+]$	$\pi \rightarrow \pi^*$, λ(ε)	CT
$(CN)_5FeL^{3-}$	10^{-5}	263 (6546)	449 (5070)
$(CN)_5FeLH^{2-}$	10^{-2}	280 (5891)	605 (3263)
$H(CN)_5FeLH^-$	0.51[b]	280 (5487)	550 (3655)

[a] μ = 0.40, T = 25 °C, Cary 15, L = 2-methylpyrazine. [b] μ = 0.61.

chemical shifts at δ 9.02 (s), 8.97 (d), and 807 (d) for bound ligands and 8.45 (s) for the free ligand.[12] For steric reasons the single isomer is most likely coordinated through the unhindered N-4 of the ring as in the 2-methylpyrazinepentaammineruthenium(II) ion.[13] Malin has measured the association constant for pyrazine and methyl-substituted pyridines with $(CN)_5Fe^{3-}$.[14] The presence of C-3 or C-4 methyl groups gave almost no effect on these association constants with $(CN)_5Fe^{3-}$. The value for pyrazine is 9.0×10^5 M^{-1}.

Association Constants for Imidazole Complexes, Ia and If. Association of 2-methylpyrazine is nearly equal to that of imidazole. An equilibrium given by eq 4 could be achieved for Ia (R = H) or If (R = CH_3) in 0.10 M NaCl solution. Equilibrium constants were determined spectrophotometrically as described in the Experimental Section for Ia and If. Using the value for the association of pyrazine for $(CN)_5Fe^{3-}$ and the equilibrium constant evaluated for eq 4 at 25 °C, the association constants were found to be 7.9×10^5 (Ia) and 4.1×10^3 (If) in agreement with the NMR ratio of about 10^2 determined by loss of bound resonance with pD titration. Apparently the



(4)

presence of the adjacent methyl group costs about 3.1 kcal/mol due to steric effects on the association in If. The measured magnitude is close to the value of 5.9 kcal/mol lower affinity of 2-methylpyridine for $B(CH_3)_3$ compared to the unhindered base.[39] However, removal of 3.1 kcal/mol of strain energy by conversion to a C-2 bound isomer is not observed by NMR. One may conclude that a C-2 coordinated imidazole would be at least 10^2 less stable than the N-3 bound form that is observed in the NMR spectrum or that the kinetic barrier to interconversion is large at pD of 7.0.

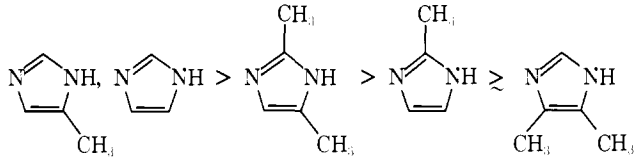
Oxygen had to be rigorously excluded during equilibrium measurements for the determinations of the association constants. On the hours time scale needed to reach equilibrium, O_2 oxidation is competitive. Loss of total Fe(II) by O_2 oxidation resulted in bleaching of the IIa absorption. IIa is not bleached by O_2 on this time scale. The resistance of IIa to O_2 oxidation implies that a reapproach to equilibrium at a lower total Fe(II) level is achieved if O_2 is admitted. The bleaching effect is caused by the shift in equilibrium from oxidation of Ia by O_2 and not by direct oxidation of IIa. Values for the association constants were determined on solutions which were not subjected to this complication. The dissociation rate of an imidazole group from Ia was determined by a scavenging ex-

Table V. Maxima of $(CN)_5FeL^{2-}$ and $(NH_3)_5RuL^{3+}$ Ions

L	$(CN)_5FeL^{2-}$ bands, nm				$(NH_3)_5RuL^{3+}$ band[4]
a	480	403	355	293	430
c	510	402	358	End abs	
d	525	403	354	292	470
e	565	403	356	290	
f	595	403	358	End abs	520
g	485	403	357	292	450[5]
h	480	410	353	293	425
CN^-	420	410	320	302, 262	

periment at high 2-methylpyrazine concentration as 5.34×10^{-4} s^{-1} ($\mu = 0.10$, 25 °C).

Preliminary data on Ie show the dissociation half-time is less than 10 s for conversion of Ie to IIa. This observation is in harmony with the broadened NMR resonances. The half-times for conversion of Ia to IIa is 1.3×10^3 s and for Ic to IIa is about 3.6×10^2 s. The slower dissociation of Ic compared to Ie is in agreement with the slightly broadened methyl resonances of Ic. By symmetry Ic, Ie, and If should possess equal steric effects on the stability of the respective ions. Assuming association constants for Ic and Ie are nearly 4.1×10^3, the value of the association constant for If, the differences suggest a steric effect on the substitution of the ligands of $(CN)_5Fe$-H_2O as well. A predicted decreasing order is obtained for the rate of ligand substitution with some uncertainty as to the exact order of e, c, and f.



(a,d > e > c ≳ If)

Imidazole Complexes of $(CN)_5Fe^{2-}$. Ia-g could be oxidized to species IIIa-g. Characterization of the composition of IIIa-g was made on the basis of three pieces of evidence: (1) the isolation of a $Zn(CN)_5Fe(imz)$ salt; (2) the presence of a titratable pyrrole hydrogen; and (3) the visible spectral properties of a long-wavelength band in the product of the H_2O_2 oxidation of Ia-g. Table V lists the band maxima observed for IIIa-g. All the complexes contain nearly constant bands at 292, 355, and 403 nm due to transitions involving only the CN^- ligand. Similar maxima are observed for $Fe(CN)_6^{3-}$. The long-wavelength band of complexes IIIa-g serves to reveal that the imidazole unit is retained on the Fe(III) center after oxidation of the corresponding Fe(II) complex.

In the absence of the spectral data the presence of the substituted imidazoles and the presence or absence of the imidazole moiety could be in doubt. Indeed the failure of Ia to obey Nernst titration behavior suggested dissociation subsequent to metal oxidation as a possible explanation.

Figure 1 shows the plot of the long-wavelength band maxima of substituted imidazole complexes of $(CN)_5Fe^{2-}$ against a similar maxima obtained from literature spectra of corresponding Ru(III) complexes. The long-wavelength transitions in the $(NH_3)_5RuL^{3+}$ species have been attributed to ligand-to-metal charge-transfer bands. The slope of 1.00 is consistent with assigning the metal oxidation state in the iron complexes as Fe(III) in accordance with the Murrel simplified equation[15] for one-electron charge-transfer states.

Figure 2 presents the spectrum of IIIa as a function of hydrogen ion. The compound IIIa was isolated by anion exchange. On titration with NaOH the spectrum altered to

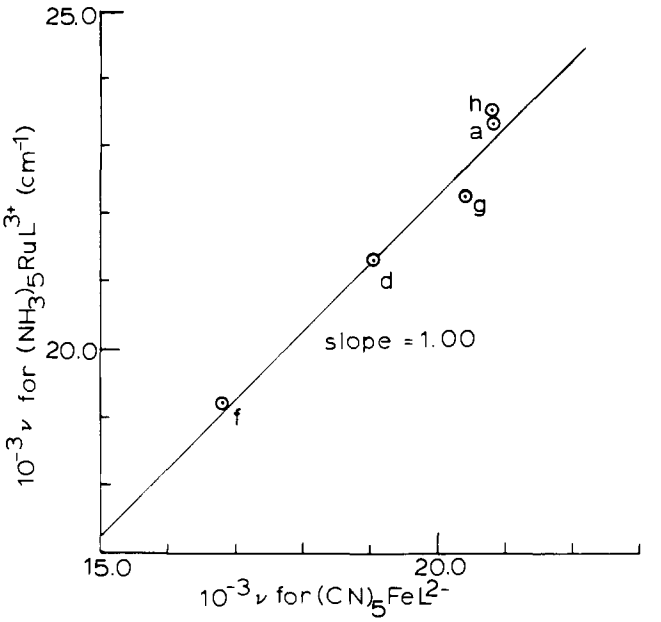


Figure 1. a = imidazole, d = 5-methylimidazole tautomer, f = 4,5-dimethylimidazole, g = L-histidine, h = *N*-methylimidazole.

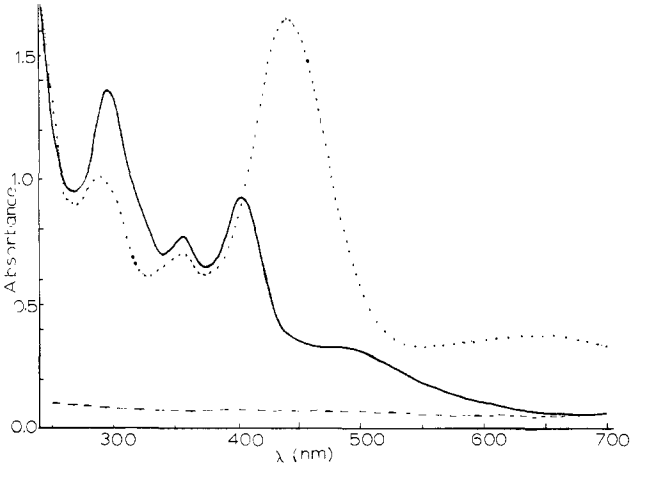
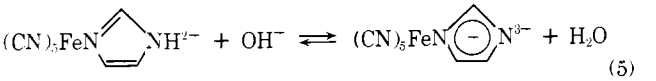


Figure 2. $[IIIa]_0 = 7.38 \times 10^{-4}$ M, satd NaCl, 1.00 cm: —, pH 6.94; . . . , pH 11.33.

provide a single maximum in the visible region at 440 nm and a growth in extinction in the 600-700 nm region. The change was reversed by addition of HCl to the former spectrum. The pK_a of the titration was established at 10.5 ± 0.1. A similar value has been reported for imidazole coordinated to Fe(III) in a porphyrin.[16] The titration behavior and associated spectral changes were not observed when the *N*-methylimidazole complex (Ih) was examined. These data establish the presence of the titratable pyrrole hydrogen shown in eq 5; the pyrrole N-H is absent in the *N*-methylimidazole case.



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Cyclic Voltammetry of $(CN)_5Fe(imz)^{3-}$ (Ia). In agreement with the non-Nernst titration behavior of Ia the system undergoes cyclic oxidation and reduction as shown by Figure 3. The peak-to-peak separation is 141 ± 5 mV at 0.135 V/s rather than the 59 mV expected for a system involving rapid reversible $1e^-$ transfer events in the III/II couple. Knowing the oxidation of Ia-g proceeds with retention of the imidazole ligand in the first coordination sphere, the irreversibility must

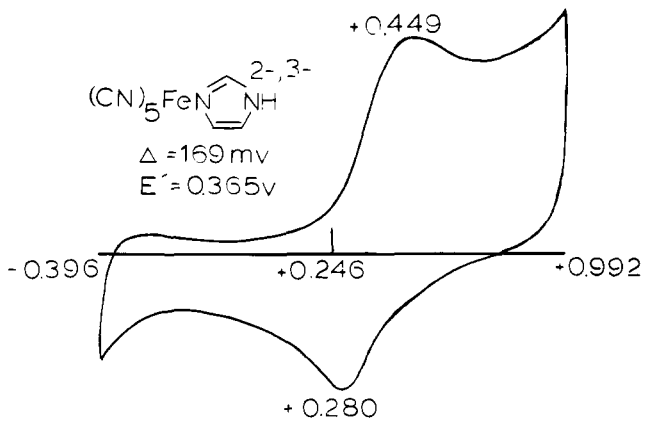
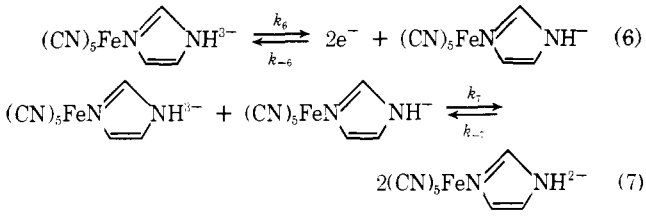


Figure 3. [Ia] = 1.00×10^{-3} M, μ = 0.10 $NaClO_4$, pH 7.0 (imidazole), T = 25 °C; cathodic current is down; voltages are corrected vs. NHE.

originate in a slow electron transfer for one of the species at the Pt electrode. The $Fe(CN)_6^{4-,3-}$ couple is also dependent on the electrode surface and inert electrolyte concentration with a peak separation of up to 120 mV at equivalent scanning rates. It is possible that the widely separated anodic and cathodic peak potentials for Ia are of the same origin as in the $Fe(CN)_6^{4-,3-}$ case. An alternate explanation is a cooperative redox event involving equilibria shown by eq 6 and 7. We are studying the oxidation of Ia by H_2O_2. Evidence for the two-



electron scheme will be discussed in a future companion paper. $(CN)_5Fe(imz)^-$ is formally Fe(IV); we cannot distinguish between an Fe(IV) complex and an isoelectronic species containing Fe(III) coordinated to an imidazole radical plus an additional proton transferred to the solvent. The step k_{-6} would appear to be the slow electron-transfer step at the Pt surface. Steps k_7 and k_{-7} are found to be rapid with second-order rate constants exceeding 10^5 M^{-1} s^{-1}.

Discussion

Our data show that histidine as well as other substituted imidazoles will coordinate to the $(CN)_5Fe^{3-}$ moiety through the N-3 position. $(CN)_5Fe^{3-}$ combines with the pyridines and pyrazines to produce a series of low-spin Fe(II) complexes that exhibit chemical behavior similar to those of $(NH_3)_5RuL^{2+}$ complexes where L is a nitrogen heterocycle.[14,17] The $(NH_3)_5RuL^{2+}$ complexes are stable in excess of what may be attributed to simple σ bonding between Ru(II) and L. The additional stability has been attributed to interaction of empty π orbitals of the heterocyclic ligand and the filled t_{2g} orbitals of Ru(II).[18,19] π back-bonding contributes about 5 kcal/mol to the interaction of pyridine with Ru(II).[13] In the imidazole, a poorer π-acceptor molecule in the N-3 bound form, a stabilization of at most 1 to 2 kcal/mol is manifest.[4,13] If back-bonding is involved in the stabilization of the ylide form by interaction of ruthenium d orbitals with a carbon p orbital, perhaps in a manner similar to the use of sulfur d orbitals in stabilizing thiazolium ylides,[20] one would anticipate that the contribution from π bonding would be less for ions of the first transition series based on smaller radial extension of 3d compared to 4d orbitals. On these grounds the association of imidazole with $(CN)_5Fe^{2-}$ would provide a rigorous test of the ylide isomerism as a possible biological control mechanism because the $(CN)_5FeL^{3-}$ complexes are favorable cases for π bonding effects and the $Fe(CN)_6^{4-}$ ion readily shows that the Fe(II) can accommodate six carbon donor ligands with back-bonding capability.

It appears as if the coordination of histidine in metalloproteins through the C-2 ring position is rather unlikely based on the behavior of Ia-g in homogeneous solution. We cannot exclude the possibility that in a biological lattice structure where dissociation, as in eq 1, is prevented, C-2 coordination might be utilized in a biological role, but these data suggest that the ylide form of histidine is at least 200 times less favorable energetically than the N-bound histidine unit for the first row transition metal ions.

The formation constant for imidazolepentaaquoiron(II) ion is 65.[21] The Fe(II) center is high spin in $(H_2O)_5Fe(imz)^{2+}$ (IVa). Compared to Fe(II) in the low-spin state, a factor of 1.2 $\times 10^4$ in favor of the low-spin association with imidazole may be calculated. The more favorable contribution to the low-spin complex of 5.5 kcal/mol is readily understood by the repulsion of the base pair of imidazole for the electron pointing directly at the ligand in the high-spin complex compared to the low-spin form where all electrons occupy orbitals that point between the ligands. The latter arrangement also provides a favorable π interaction with imidazole *even* if this effect is small. The increased stability of association of low-spin Fe(II) for imidazole could contribute to the stabilization of the diamagnetic nature of hemoglobin after oxygenation to about 5 kcal/mol. Deoxygenated hemoglobin is paramagnetic, and it has been suggested that the histidine in the 5 position moves closer to the heme iron when oxygenation occurs.[22]

Titration of the pyrrole proton occurs at a pK_a greater than 11 for Ia; a value of >12 has been observed for the $(NH_3)_5Ru(imz)^{2+}$ (Va) ion.[32] The association of imidazole is approximately 0.75 kcal/mol less favorable for $(CN)_5Fe^{3-}$ than for $(NH_3)_5Ru^{2+}$. Since this is close to the value of the back-bonding contribution in Va, one must conclude that π back-bonding to imidazole makes a rather small contribution to the stability of the Fe(II) complex when imidazole acts as a π acceptor. On the other hand if electronic demand of a trans ligand, such as O_2 in hemoglobin, is large the π donor capacity of imidazole may interact through the t_{2g} system to stabilize the Fe(II) state through charge dispersal and conformation changes in the protein structure.[1] Ligand-to-metal one-electron charge-transfer states account for the long-wavelength band in the spectra of IIIa-g. The position of the band shifts to lower energy with increased substitution of electron donating groups on the imidazole ring. The effect is largest for substitution on the C-4 position nearest N-3 coordinated to iron with additional assistance on C-5 methylation. The C-2 substitution produces the smallest effect; the C-2 carbon is electron poor on the basis of the ^1H NMR spectra of the free ligand. The C-2 position is least likely to transmit inductive effects by electron donor groups. It might be expected that an inductive effect would be largest through the σ structure where the C-4 substituent has an advantage.

Approximately the same inductive stabilization of Fe(III) is obtained by 4-methylimidazole as by histidine. Both are coordinated as the "5-methyl" tautomer and frozen into this configuration according to the NMR data of Table III.

Ligand exchange on $(CN)_5FeL^{3-}$ complexes proceeds by a dissociative mechanism given by eq 8-10 where L and L′ are the exchanging ligands.[17]

$$Fe(CN)_5L^{3-} \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} Fe(CN)_5^{3-} + L \qquad (8)$$

$$Fe(CN)_5^{3-} + H_2O \overset{K_9}{\rightleftharpoons} Fe(CN)_5H_2O^{3-} \qquad (9)$$

$$Fe(CN)_5^{3-} + L' \underset{k_{-9}}{\overset{k_9}{\rightleftharpoons}} Fe(CN)_5L'^{3-} \quad (10)$$

Equations 8-10 yield a first-order expression in $(CN)_5FeL^{3-}$ for the conversion to $(CN)_5FeL'^{3-}$ with an observed rate constant given by eq 11;

$$k_{obsd} = \frac{k_8k_9[L'] + k_{-8}k_{-9}[L]}{k_{-8}[L] + k_9[L']} \quad (11)$$

k_{obsd} is the approach to equilibrium value at high L′ as in the scavenging experiment with 2-methylpyrazine. Using values of k_9 and k_{-9} determined for pyrazine and limits to k_{-8} established by the flow experiments of Malin, a value for the dissociation rate of imidazole from the $Fe(CN)_5^{3-}$ unit is calculated as 6.1×10^{-4} s^{-1}. Combined with the association constant for Ia, a rate of substitution of imidazole on $Fe(CN)_5^{3-}$ is found to be 484 M^{-1} s^{-1}, in excellent agreement with a limiting range between 3×10^2 and 6×10^2 M^{-1} s^{-1} observed with other small heterocyclic nitrogen ligands.[17]

The behavior of imidazole coordinated to the Fe(III) state in IIIa before and after ion exchange suggests a large association constant for IIIa. Under pseudo-first order conditions, addition of imidazole to $(CN)_5FeOH_2^{2-}$ occurs with a specific second-order rate constant of 0.31 M^{-1} s^{-1}. Comparison of substitution on $Fe(CN)_5^{3-}$ and $Fe(CN)_5^{2-}$ shows a rate factor in favor of Fe(II) of about 1.6×10^4, in good agreement with the water exchange rate ratio between $Fe(H_2O)_6^{3+}$ and $Fe(H_2O)_6^{2+}$ of 1×10^3.

The overall behavior of the $Fe(CN)_5^{2-}$ moiety toward imidazole shows several close parallels to ferrimyoglobin and other iron porphyrins and biopolymers. Incorporating an upper limit value for the dissociation of IIIa based on our ligand exchange studies, a minimum association constant for IIIa was found to be 5.1×10^2 M^{-1}. The association constant of imidazole and ferrimyoglobin has been found to be 1.6×10^2 M^{-1}.[21] Ferrimyoglobin and IIIa exhibit similar titration behavior of the pyrrole hydrogen having pK_a values of 10.34 and 10.5, respectively. The shift in the pK_a of the pyrrole hydrogen is not as large as has been observed in $(NH_3)_5Ru(imz)^{3+}$, suggesting that CN^- ligands or porphyrins quench some of the positive charge on the Fe(III) center compared to Ru(III). A charge neutrality of this type has been considered for many years to explain aspects of the chemistry of $Fe(CN)_6^{3-}$ compared to $Fe(CN)_6^{4-}$ and the principle of lesser than a full 3+ iron charge has been substantiated by ESCA.[23]

The rate of dissociation of imidazole from Ia (6.1×10^{-4} s^{-1}) is in reasonable agreement with the exchange or dissociation of small molecules from ferromyoglobins, hemoglobins, and other low-spin Fe(II) complexes having approximately D_{4h} symmetry. The dissociation rates of O_2 from myoglobins have the highest values with a range of 10-70 s^{-1}.[24] However, the 70 s^{-1} is atypical. Most of the measured values of k_d for O_2 from myoglobins are closer to 10 s^{-1}. Isocyanides and CO dissociate with k_d values of about 0.8 and 0.04 s^{-1}, respectively, for myoglobins and hemoglobins.[24] Dissociation of CO and CN^- from cytochrome a_3 are 0.02 and 0.08 s^{-1}.[25] Gibson reports that the first cyanide is lost at a rate of 22 s^{-1} from a dicyanoheme.[25] Rose et al. have observed that imidazole is slow to exchange on the NMR time scale on $Fe(TIM)(imz)_2^{2+}$, where TIM = 2,3,9,10-tetramethyl-1,4,8,11-tetraazacyclotetradeca-1,3,8,10-tetraene.[26] Vaska reports that dissociation of pyridine from $Fe(dmg)(py)_2$ (dmg = dimethylglyoxamato ligand) has a rate constant of 4.22×10^{-3} s^{-1}, while CO dissociation from Fe(dmg)(py)(CO) is 10^2 slower.[27] A referee has raised the issue of why the rate for dissociation of imidazole from $Fe(CN)_5^{3-}$ is slower (by about 10^3) than in the hemoproteins. Stynes, Stynes, James, and Ibers have shown that the globin environment may alter the effective overall association constants by a factor of 50 in comparing simple heme complexes $(pip)_2$FeTPP, $(pip)_2$Fe(protoporphyrin IX), and globins.[28] It is difficult to decide how this effect is predominantly manifest: whether k_d is decreased, k_f increased, or by a compensation in both rates. Major differences between the low-spin environment of five cyanide ligands and that of a porphyrin ring plus a histidine moiety include (1) the charge on the axial CN^- ligand, (2) π donor and acceptor properties of CN^- compared to imidazole, (3) possible spin-state thermal equilibria accessible for hemoproteins that are not accessible for $Fe(CN)_5^{3-}$, and (4) the porphyrin ring donor and acceptor properties compared to four cyanide ions.

More information on the nature of donor and acceptor nature of a porphyrin compared to axial ligands has been obtained from ferrimyoglobin or ferric porphyrin complexes such as Fe(TPP)Cl and their imidazole complexes $Fe(TPP)(imz)^+$ and $Fe(TPP)(imz)_2^+$ than on Fe(II) derivatives.[29,30] LaMar and Walker have indicated that for low-spin ferric porphyrin complexes, the predominant charge transfer involves M ← P from the highest filled porphyrin π MO.[29] The low-spin Fe(II) complexes should favor M → L π charge transfer; however, Straub et al. have demonstrated the tendency of imidazole to stabilize ferric porphyrins compared to the ferrous state.[31] This suggests the π-acceptor capacity of imidazole is small. This result is in agreement with our observations on the stability of $(CN)_5Fe(imz)^{3-}$. Our results with $(CN)_5Fe(imz)^{2-}$ (IIIa), show that imidazole is a better π donor than CN^- toward the Fe(III) center, while imidazole is less able to stabilize the Fe(II) state compared to CN^-. This latter effect may well be of a mechanistic origin since the pyrazine complex has comparable kinetic stability and is not sensitive to O_2. Parallel reactivities are exhibited by the Ru(II) ammines. The spectral band at 381 nm for Ia indicates some degree of allowable M → L π charge transfer for the Fe(II) state with imidazole as an acceptor. This MLCT state is energetically more accessible than if CN^- is the sixth ligand and implies increased bonding for the charge separated excited state. An uncertainty of the interpretation is introduced by the variation of CN^- in its σ-donor-π-acceptor properties as a function of the remaining ligand environment. Fenske et al. have shown that cyanide is a better π acceptor and weaker σ donor in $Mn(CN)_6^{5-}$ than it is in $Mn(CO)_5CN$.[33] CO, on the other hand, remains relatively constant as a σ donor but is affected only as a π acceptor by the other ligands.[34] There would not seem to be sufficient data at hand to specify how imidazole is affected in these properties by comparison between porphyrin and cyanide environments. However, the spectral observations for Ia suggest that the π-acceptor capacity of imidazole may increase trans to CN^- somewhat like the effect for CO.

Meyer and Whitten recently showed from cyclic voltammetry data for a series of Fe, Ru, and Os porphyrins that axial ligands are able to modulate the relative energy levels of d_{z^2} and the d_{xz}, d_{yz} pair.[35] When the axial ligands are CO and nitrogen base heterocycle (imidazole or pyridine), the d_{xz} and d_{yz} are the highest filled metal orbitals in M(P)(CO)(L). The question of importance then becomes how rapidly does the d_{z^2} orbital sink compared to the elevation of the d_{xz} and d_{yz} pair which are available for π-bonding (M → L) when the ligand trans to a leaving group is CN^- as in Ia or imidazole in the hemoproteins?

On the basis of these diverse sources of information in which secondary effects may cloud definitive conclusions, it would seem that for the porphyrins, which are themselves π donors, a ligand trans to a good π donor, CN^-, or imidazole is labilized. CO as a better π acceptor than either CN^- or imidazole should lower d_{z^2} which is directed at the leaving group of ligand exchange. CO should then give lower values for k_d in agreement with Vaska's data.[27] The dissociation behavior of Ia is normal for the Fe(II) state as established by the model complexes of Rose and Vaska where k_d values are within an order of mag-

nitude of the k_d for Ia. The inclusion of thermal spin-state equilibria would not seem to be necessary to account for the factor of 10^3 in dissociation rates with a factor of 50 due to the protein environment and another factor of 20 originating in small variation in base strength and π donor/acceptor properties of different ligands. There are solvation effects originating from different experimental media which were used to deduce these trends. The effects of solvation on the entropy of the reactions are able to contribute a factor of 10 in rates.[36]

Fe(IV) intermediates have been postulated in the reactivity of horse-radish peroxidase[37] and the cytochrome system.[38] Our observations also support a possible role for two-electron events for low-spin iron centers either by two-electron changes in the metal center itself or with concerted ligand oxidation as an accessible chemical pathway for biological activity.

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Electrostatic Potentials of Proteins. 1. Carboxypeptidase A

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Abstract: We present a new approach to examining the qualitative features of protein structure and small molecule-protein interactions. The electrostatic potential, which is a useful guide for small molecule interactions, is examined for carboxypeptidase A (CPA), its apo derivative, and for the CPA-Gly-Tyr complex. Ab initio wave functions are used to represent the individual residue partial charges. An *o*-OH-Gly-Tyr analogue is predicted to bind more strongly to CPA than the natural Gly-Tyr substrate.

Since there are an ever increasing number of proteins for which high resolution x-ray data are becoming available, it is of interest to examine their electronic as well as conformational properties. In this report, we present a study of the electrostatic potential of carboxypeptidase A (CPA), one of the best resolved and characterized globular proteins. Quiocho and Lipscomb[1] have thoroughly discussed the structure and mechanism of action of CPA in light of x-ray structural studies.[2] One of our objectives in this study is to understand the important contributing factors to enzyme-small molecule binding. CPA is a good choice for these studies because its x-ray pattern is known to high resolution (2 Å) and because the structure of an enzyme-substrate complex (Gly-Tyr) of CPA has been determined.[3]

Briefly, CPA is an exopeptidase, i.e., it cleaves peptide bonds at the carboxyl terminal end of the peptide chain. It also has catalytic activity toward esters and requires divalent metal for its catalytic activity (Zn^{2+} in the native enzyme) and a pH for optimum catalytic activity of 7.5. In addition, upon binding of a substrate the enzyme undergoes a remarkable conformational change involving at least three side chains of CPA (Arg 145, Tyr 248, and Glu 270). This altered structure we will henceforth call "bound" CPA to distinguish it from the native or unbound enzyme. Many workers have probed the specificity of CPA by allowing it to react with a variety of different substrates.[4] These studies show that at least the five terminal peptides of the substrate influence the observed kinetic parameters.[5] This is consistent with the finding from the x-ray structure that the active site pocket (~18 Å in length) has room for at least five peptides.[1,6]

Since the publication of a paper by Bonaccorsi et al.,[7] there has been increasing interest in electrostatic potential maps as