Volume 21
Number 3
March 1982

Inorganic Chemistry

Contribution from the Department of Chemistry, University of Rochester, Rochester, New York 14627

Outer-Sphere Electron-Transfer Reactions of the Isolated Active-Site Heme Octapeptide from Cytochrome *c*

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Received May 5, 1981

The electron-transfer reactivity of the isolated cytochrome *c* active-site peptide (heme octapeptide) has been studied. Two separate approaches have been used: photochemical electron transfer from excited-state ruthenium bipyridyl homologues and NMR measurements. Both techniques provide a consistent estimate of the self-exchange rate for the heme peptide: $k_{11}^{OP} > 10^6\ M^{-1}\ s^{-1}$ (μ = 0.1 M). This rate appears constant for derivatives with pyridine, imidazole, or methionine as axial ligands. The heme peptide self-exchange rate exceeds the self-exchange rate for cytochrome *c*, which is estimated as $k_{11}^{cyt\ c} \simeq 10^3$–$10^4\ M^{-1}\ s^{-1}$. The results show that incorporation of a prosthetic group in a metalloprotein need not increase the basic reactivity of the metal and may indeed decrease it. Possible explanations for this decrease are considered.

Introduction

A central question in inorganic biochemistry is "How does a protein influence the reactivity of the associated metal center?" In this paper, the electron-transfer reactivity of cytochrome *c* is compared with a "model" system, the heme octapeptide, which is an isolated active-site peptide of cytochrome *c* (Figure 1). This peptide is clearly an ideal model system, since the heme *c* substituents, several specific heme amino acid contacts, and histidine axial coordination are preserved in the isolated active site. The other axial ligand site can be occupied by methionine, as in native cytochrome *c*, or by other ligands (CN^-, pyridine (py), imidazole (im)) as desired.[1,2] This heme peptide has been well characterized and many of its properties are known.[1-3] Differences have been found between the redox potentials[1] and between electronic structures[3] of cytochrome *c* and the isolated active site. However, a fundamental comparison of electron-transfer rates has not been reported.

We here report the first comparison of the intrinsic electron-transfer reactivity (e.g., self-exchange rates) of the heme site between the native protein and the isolated active site, involving a novel application of excited-state electron transfer.

Materials and Methods

Materials. The heme octapeptide (OP) of cytochrome *c* was isolated as previously described.[2] Amino acid analysis agreed well with the expected values.

Ruthenium pyridyl homologues, $Ru^{II}L_3$ (L = bipyridine (bpy), 4,7-dimethylphenanthroline, 5-chlorophenanthroline (5-Cl-phen), bipyrimidine) were prepared as previously described.[4,5]

Methods. Magnetic resonance was attempted in order to measure the self-exchange rates of OP independently. A known concentration (ca. 10^{-4} M) of the OP–pyridine complex was reduced in an anaerobic electrolysis cell and transferred by syringe to a degassed NMR tube containing an oxidized OP–pyridine solution of known concentration. NMR spectra of the pure oxidized, pure reduced, and mixed solutions were obtained on a 100-MHz JEOL PFT-100 or a 400-MHz Bruker-400 instrument.

Excited-state reactions were monitored by luminescence quenching. Emission of ruthenium bipyridyl homologues was monitored by using a Perkin-Elmer MPF44A fluorimeter. Aliquots of octapeptide solutions were added with a gastight syringe.

Heme axial-ligand effects were examined for pyridine, imidazole, and *N*-acetylmethionine. Identical excess ligand concentrations were maintained in both the ruthenium solutions and the octapeptide solution ([pyridine] = 0.05 M, [imidazole] = 0.05 M, [*N*-acetylmethionine] = 2.5 M). Solutions of the ligands alone did not quench the ruthenium emission.

Electron-transfer quenching was established by photolyzing an anaerobic solution containing ruthenium bipyridyl, octapeptide, pyridine, and EDTA. Continuous irradiation showed clean reduction of the $(OP)Fe^{III}py$.

Results

Photoinduced Electron Transfer. Many previous studies of protein electron transfer have used stopped-flow methods as a primary technique.[13] However, the combination of the unfavorable reduction potential ($E° < -0.1$ V), extreme oxygen sensitivity, and rapid self-exchange of the heme octapeptide makes conventional flow techniques difficult or impossible to apply. Photoinduced electron transfer offers a simple and potentially powerful way to circumvent the constraints of the flow experiments. Homologues based on ruthenium(II) bipyridyl readily undergo excited-state electron transfer over a wide range of excited-state potentials.[4,6] Since electron transfer can be detected by quenching of ruthenium emission, elaborate precautions need not be taken to protect the redox products from air oxidation. (Air was routinely excluded, however, to prevent partial quenching of the ruthenium excited state.)

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0020-1669/82/1321-0847$01.25/0

Table I. Electron-Transfer Quenching Parameters for the Reaction

$$Ru^*L^{2+}OP^{II}L^1 \rightleftharpoons OP^{II}L^1 + RuL_3^{3+}$$

L	L^1	τ RuL_3, μs	$E^{\circ a}$, V	k_{obsd}, M^{-1} s^{-1} [b]	k_{11}^{OP}, M^{-1} s^{-1} [c]
bipyridine	pyridine	0.6	0.7	3×10^{10}	1×10^6
	imidazole	0.6	0.6	3×10^{10}	1×10^6
	methionine	0.6	0.76	5×10^{10}	2×10^6
5-chlorophenanthroline	pyridine	1.0	0.6	4×10^{10}	2×10^6
	imidazole	1.0	0.5	2×10^{10}	1×10^6
	methionine	1.0	0.66	4×10^{10}	1.7×10^6
bipyrimidine	pyridine	0.08	0.27	1×10^{10}	4×10^6
	imidazole	0.08	0.17	5×10^9	3×10^6
	methionine	0.08	0.31	2×10^{10}	4×10^6

[a] $E^\circ = E^\circ_{Ru^*L_3 \rightarrow RuL_3^{3+}} + E^\circ_{OP^{II}L \rightarrow OP^{II}L}$; $E^{\circ *}_{Ru(bpy)_3} = 0.86$ V;[6] $E^\circ_{OPim} = -0.22$ V,[1] $E^{\circ *}_{Ru(bpm)_3} = 0.40$ V;[5] $E^\circ_{OPpy} = -0.12$ V;[1] $E^{\circ *}_{Ru(5\text{-}Clphen)_3} = 0.78$ V;[6] $E^\circ_{OP(methionine)} = -0.08$ V.[1] [b] $k_{obsd} - k_{SV}/\tau$, where k_{SV} is the Stern-Volmer constant obtained from static quenching and τ is the Ru* lifetime. [c] $k_{11}^{OP} = (k_{obsd}^2/k_{22}^{Ru}k_{12}f)$; $k_{12} = 10^{E^\circ/0.059}$; $\ln f = (\ln k_{11}k_{22}/z^2)$; $k_{22} \simeq 10^9$ M^{-1} s^{-1} (ref 6).

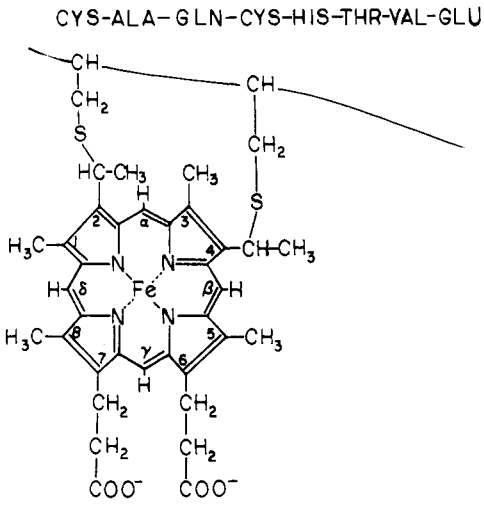

Figure 1. Schematic representation of the heme octapeptide (cysteinylalanylglutamanylcysteinylhistidylthreonylvalylglutamate).

Thus, this technique has recently been applied in studies of the cross reactions of cytochrome *c*[7] and the blue copper proteins.[8]

The present studies underscore the utility of the technique. It is crucial in these quenching experiments to establish that quenching indeed occurs by electron transfer and not energy transfer. An electron-transfer path was demonstrated for octapeptide quenching by irreversible reaction of the Ru^{III} photoproduct with EDTA, thereby "trapping" the reduced octapeptide. Such competitive reactions have been studied in detail, in connection with photocatalytic water reduction.[9,10] The system employed is given in eq 1 and 2. Thus, when a

$$Ru(bpy)_3^{*2+} + OP^{III} \rightarrow OP^{II} + Ru(bpy)_3^{3+} \quad (1)$$

$$Ru(bpy)_3^{3+} + EDTA \rightarrow Ru(bpy)_3^{2+} + CH_2O + CO_2 + \text{ethylenediaminetriacetate} \quad (2)$$

system containing 0.01 M EDTA, 1×10^{-5} M $Ru(bpy)_3$, and 3×10^{-5} M $(OP)Fe^{III}py$ is irradiated with visible light, the Fe^{III} peptide is quantitatively reduced within 5 min to Fe^{II} (Figure 2). Therefore, the OP quenching of ruthenium luminescence indeed occurs by electron transfer. Stern–Volmer quenching data for electron-transfer deactivation of RuL_3 homologues (L = bipyridine, 5-chlorophenanthroline, bipyrimidine) by several octapeptide derivatives (imidazole, pyridine, *N*-acetylmethionine) are shown in Figure 3. The results of all the experiments are summarized in Table I. The standard relative Marcus equation is used to calculate the OP

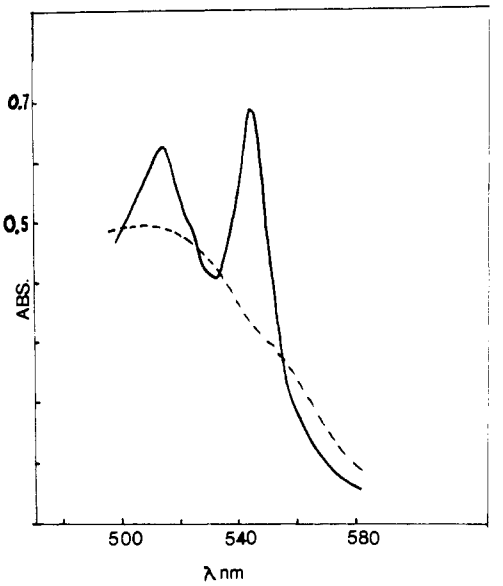

Figure 2. Visible spectra in the α,β-band region of the heme octapeptide/$Ru(bpy)_3^{2+}$/EDTA photoreduction system: (---) absorbance before irradiation ($[Ru(bpy)_3^{2+}] = 1 \times 10^{-5}$ M, $[Fe^{III}OP] = 3 \times 10^{-5}$ M, [EDTA] = 0.01 M, pH = 7 (~0.1 M Bistris buffer)); (—) absorbance after 5 min of irradiation with a 200 W projector bulb. Irradiation light was restricted with a 400-nm sharp-cutoff filter and an IR filter. Radiant intensity was $\sim 10^{-7}$ einstein/s, determined by Reineckes salt actinometry.[9]

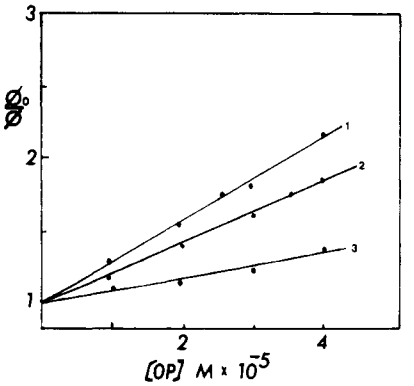

Figure 3. Stern–Volmer plots (ϕ_0 = emission intensity at 620 nm without quencher, ϕ = intensity at each quenching concentration) for electron-transfer quenching of RuL_3^{2+} (L = 5-chlorophenanthroline (1), bipyridine (2), bipyrimidine (3)) by $(OP)Fe^{III}$ imidazole. Similar plots are obtained for the methionine and pyridine adducts (Table I).

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self-exchange rate, k_{11}: $k_{12} = (k_{11}k_{22}k_{12}f)^{1/2}$, where k_{22} is the excited-state ruthenium^{II} exchange rate; $k_{22} \sim 10^9$ M^{-1} s^{-1}; K_{12} is the equilibrium constant calculated from the OP and Ru^{II*} redox potentials, and f is defined in Table I. The most accurate estimate of k_{11} should be obtained from the reactions of $OP^{III}L$ with $[Ru(bpm)_3]^{2+*}$, for which the driving force is minimal ($E \sim 0.16$ V; Table I). The data in Table I are consistent with $k_{11} > 10^6$ M^{-1} s^{-1}. Assuming $k_{11} = 10^3$ M^{-1} s^{-1}, as in cytochrome c, leads to the prediction that $k_{obsd} = (10^3 10^9 10^{3.1})^{1/2} = 10^7$ M^{-1} s^{-1}, which is almost 2 orders of magnitude smaller than the observed value ($k_{obsd} \geq 10^9$ M^{-1} s^{-1}). The value $k \simeq 10^6$ M^{-1} s^{-1} is also consistent with the lower limit for self-exchange established by NMR spectroscopy for OPpy (vide infra).

Finally, this value is similar to, but smaller than, an estimate by Pasternak[17] of the self-exchange rate of a low-spin synthetic iron porphyrin, hydroxyiron tetrakis(methylpyridyl)porphyrin. For this (fully exposed) synthetic porphyrin, the estimated self-exchange rate is $k_{11} > 10^7$ M^{-1} s^{-1}.[17]

The calculated values of k_{11}^{OP} were affected only minimally by the nature of the axial ligand (imidazole, pyridine, and methionine). However, the pyridine and methionine values must be interpreted cautiously, since the larger driving forces associated with these reactions ($E^\circ \geq 0.25$ V) increase the quench rate to near the diffusion limit.

Magnetic Resonance. In principle, NMR techniques can provide a direct measure of self-exchange rates, under specific conditions.

Line-shape analysis can be used for reactions in the fast-exchange region ($\tau^{-1} << (\nu_{Ox} - \nu_{Red})$). This analysis is precluded for the present case by the ancillary line broadening due to rapid axial ligand exchange from the OP.

For slower reactions, direct T_1 measurements (saturation recovery, 180–τ–90) may be appropriate since $T_{1\,Ox} << T_{1\,Red}$, which is due to efficient paramagnetic relaxation of the oxidized (OP)Fe^{III}. This method is most applicable in the slow- to intermediate-exchange region: $\tau > 2^{1/2}/2\pi(\nu_{Ox} - \nu_{Red})$, where ν_{Ox} and ν_{Red} are the frequencies of the oxidized and reduced species.

In any event, an estimate of the exchange rate is provided by simply determining whether the fast- or slow-exchange limit is obeyed.

In the slow-exchange limit, ($\tau > 2^{1/2}/2\pi(\nu_{Ox} - \nu_{Red})$), the spectrum of a solution that contains both OP_{Ox} and OP_{Red} will appear to be a superposition of the isolated oxidized and reduced spectra.

By contrast, in the fast-exchange limit, only a single set of resonances will be observed. The shifts (δ) of these resonances will be weighted by the relative mole fractions, N, of oxidized and reduced OP: $\delta_{obsd} = N_{Ox}\delta_{Ox} + N_{Red}\delta_{Red}$.

As shown in Figure 4, only a single set of resonances is observed in mixtures of (OP)Fe^{II}py and (OP)Fe^{III}py. Furthermore the frequencies of these peaks are weighted toward the excess component. Thus, OP electron exchange is fast on the NMR time scale under our conditions. This observation requires a minimum self-exchange rate of $k_{11} \geq 10^5$ M^{-1} s^{-1}. A more quantitative analysis is precluded by ligand-exchange contributions to the line shape. Nonetheless, this estimated lower limit agrees with our independent determinations of k_{11}^{OP} by luminescence quenching.

Discussion

Two independent methods have been used to estimate the electron-transfer self-exchange rate constants of the active-site heme octapeptide in several low-spin ligand forms. The results are consistent with a self-exchange rate constant of ca. 10^6 M^{-1} s^{-1}. Within the limits of the data, this rate constant appears to be insensitive to axial ligation, whether via nitrogen (pyridine) or sulfur (methionine).

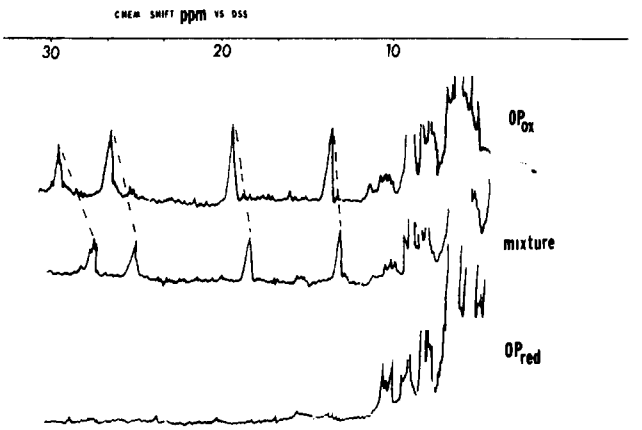

Figure 4. 100-MHz ^1H NMR spectra of the heme octapeptide (10^{-3} M total heme). The horizontal axis gives chemical shift in ppm relative to DSS (4,4-dimethyl-4-silapentanesulfonate): (a) oxidized; (b) mixture 90% oxidized + 10% reduced; (c) reduced.

More surprisingly, the self-exchange rate constant of the isolated active site exceeds that of the holoprotein ($k_{11}^{OP} \simeq 10^6$ M^{-1} s^{-1}, $k_{11}^{cyt\,c} \simeq 10^3$ M^{-1} s^{-1}). An analogous result recently has been reported. Holm and co-workers determined the self-exchange rates by NMR for synthetic Fe_4S_4 active sites.[11] The measured self-exchange rate constant of $\simeq 10^6$ M^{-1} s^{-1} for the models far exceeds the values for the native ferredoxins ($<10^3$ M^{-1} s^{-1}). At least for these two classes of proteins, then, the protein structure does not appear to enhance reactivity, as previously suggested,[12] but rather decreases it. At least two explanations for this effect may be proposed.

One explanation follows from the extensive studies of Gray and co-workers.[13] They found that the reactivity of cytochrome c with inorganic redox reagents roughly correlates with the stereochemical properties of these reagents. This correlation was proposed to reflect the relative accessibility off the protein redox site to these reagents. This idea is further supported by the recent studies of Sykes and Margoliash,[14] which support the cytochrome c heme edge as the reaction site for inorganic reagents.

It is clear that the heme peptide should be far more accessible when isolated than when incorporated into the protein. The enhanced reactivity of the exposed peptide is predicted by the accessibility model and thereby supports this model. A similar argument has been offered for the Fe_4S_4/ferredoxin comparison.[11]

While this explanation is attractive, alternatives could be offered. For example, in the original Marcus formulation,[15] the ultimate rate of electron transfer depends on the rate of reorganization of the surrounding medium (precursor and successor complex formation). With the isolated active-site models, this reorganization occurs in the bulk solvent, while for the holoproteins, the protein structure itself must rearrange. Small but well-defined changes occur in cytochrome c on reduction.[16] Since the time scale for these changes is unknown, protein reorganization may retard the overall rate of electron transfer.

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In summary, two independent methods have been used to measure the self-exchange rate of several ligand derivatives of the active-site heme peptide from cytochrome *c*. These methods give a consistent estimate of $k_{11}^{OP} = (3 \pm 1) \times 10^6$ M^{-1} s^{-1}, which significantly exceeds the rate constant for native cytochrome *c*: $k_{11}^{cyt\,c} \simeq 10^3$–$10^4$ M^{-1} s^{-1}. This rate difference between the protein and isolated active site is consistent with the accessibility model proposed by Gray,[13] but alternative explanations cannot be excluded. Finally, excited-state electron transfer appears to offer a powerful probe of the electron-transfer reactions of metalloproteins and peptides which are difficult to study by conventional means. Further studies in this vein are in progress.[7,8]

Acknowledgment. This work was supported by Grant HL21461 from the NIH and by the unrestricted support of a Dreyfus Teacher-Scholar Award and an A. P. Sloan fellowship to G.M. We are grateful to Harry Gray for helpful discussion.

Registry No. Cytochrome *c*, 15710-60-8; $Ru(bpy)_3^{2+}$, 15158-62-0; $Ru(5\text{-Cl-phen})_3^{2+}$, 47860-47-9; $Ru(bpm)_3^{2+}$, 80263-32-7; pyridine, 110-86-1; imidazole, 288-32-4; methionine, 63-68-3.

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Kinetics and Mechanism of the Equilibration Reactions of Diaquochromium(III)–Schiff Base Derivatives, Cr(Schiff base)$(H_2O)_2^+$, and Their Conjugate Bases with Thiocyanate, Azide, Imidazole, Pyridine, and Nicotinic Acid as Ligands

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Received March 6, 1981

The kinetic studies of the reactions of the complexes Cr(Schiff base)$(H_2O)_2^+$ where Schiff base is either *N,N'*-ethylenebis(salicylidenimine), viz., salen, or *N,N'*-ethylenebis(acetylacetonimine), viz., acacen, reveal high aquo ligand substitution rates. The Schiff base complexes Cr(Schiff base)$(H_2O)_2^+$ and their conjugate bases equilibrate with a variety of nucleophiles, X^{n-}, viz., thiocyanate ion, azide ion, pyridine, imidazole, and nicotinic acid. The forward rates for the equilibration reactions of X^{n-} with either Cr(salen)$(H_2O)_2^+$ or Cr(acacen)$(H_2O)_2^+$ are independent of the nature of X^{n-}. The invariance of the forward rate constants and a linear free energy relation between log (aquation rate constants) and log (equilibration constant) with a slope of 0.94 ± 0.05 for the reactions of Cr(salen)$(H_2O)_2^+$ support a dissociative interchange (I_d) mechanism for the aquo cation. On the other hand, the forward rate constants for the conjugate base of Cr(salen)$(H_2O)_2^+$ exhibit a marked dependence on the nature of the incoming nucleophiles, X^{n-}. On the basis that the conjugate base exhibits a probable five-coordinate structure in solution, a bimolecular addition mechanism has been proposed for the reactions with X^{n-}. The study reports one of the few examples in the aqueous chemistry of Cr(III) wherein a high kinetic lability resulting from ground-state distortion has been demonstrated.

Introduction

Whereas a vast majority of aquo ligand substitution reactions of chromium(III) complexes has established the kinetic inertness of the metal ion,[1-4] ground-state distortion has recently been shown to give rise to kinetic lability in Cr(III)–Schiff base complexes.[5] The X-ray structure of the diaquochromium(III) complex, Cr(salen)$(H_2O)_2^+$ as a chloride salt reveals a distortion in the H_2O–Cr–OH_2 axis,[6-8] and such a distortion has led to the substitution of an aquo ligand by NCS^- or N_3^- in the stopped-flow time scale.[5] We have recently reported the synthesis and characterization of the perchlorate salt of Cr(salen)$(H_2O)_2^+$ as well as of the new complexes Cr(acacen)$(H_2O)_2^+$ and Cr(salophen)$(H_2O)_2^+$.[9] Although the Schiff base ligands salen, acacen, and salophen have strong structural resemblances, with cobalt(III), they have been known to form either five- or six-coordinate complexes depending on the nature of the tetradentate[10] ligand. The kinetic studies of the aquo ligand substitution reactions of chromium(III)–Schiff base complexes where Schiff base is either salen or acacen or salophen are of interest. Further with the salen derivative of chromium(III), under alkaline conditions, a complex of the formulation of Cr(salen)OH has already been isolated and characterized.[6] There have been recent reports on the interesting features of the mechanisms of the reactions of five-coordinate complexes.[11] The kinetics and mechanism of the reactions of Cr(Schiff base)$(H_2O)_2^+$ complexes (where Schiff base = salen or acacen or salophen) and of their conjugate bases with a variety of nucleophiles $X^{n-} = NCS^-$, N_3^-, pyridine (py), imidazole ($C_3H_4N_2$) and nicotinate ion (3-py-COO^-) have, therefore, now been reported.

Experimental Section

Materials. The complexes Cr(Schiff base)$(H_2O)_2ClO_4$, where Schiff base is salen, salophen, or acacen, were prepared by the adaptation of the procedures already described for the chloride salt of the salen derivative and characterised as already described.[6,9] Lithium thiocyanate and lithium azide were prepared from KNCS and KN_3 and $LiClO_4$. The other reagents pyridine, imidazole, and sodium nicotinate used were of reagent grade and were used without further purification. The concentrations of the solutions of the ligands investigated were analysed by standard methods.[12]

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