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Transition-Metal Compounds as New Reagents for Selective Cross-Linking of Proteins. Synthesis and Characterization of Two Bis(cytochrome *c*) Complexes of Platinum

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This study shows, for the first time, that proteins can be cross-linked selectively via transition-metal compounds to form stable protein complexes. Incubation of horse cytochrome *c* (designated cyt) with reagents $PtCl_4^{2-}$ and *trans*-$[Pt(2\text{-Fpy})_2Cl_2]$ under mild conditions yields stable diprotein complexes *trans*-$[PtCl_2(cyt)_2]$ and *trans*-$[Pt(2\text{-Fpy})_2(cyt)_2]$, respectively (2-Fpy is 2-fluoropyridine). The complexes are purified and characterized chromatographically. The protein molecules are coordinated to the Pt(II) atom through the thioether side chains of their respective Met 65 residues. Spectroscopic and electrochemical measurements indicate that, except for the slightly increased abundance of the high-spin form of ferriheme, the structural and redox properties of the cytochrome *c* molecules remain unaltered upon cross-linking. The diprotein complexes are stable indefinitely under ordinary conditions and yet they can be cleaved, and the native protein restored, in a mild reaction. Platinum compounds hold promise as selective and versatile reagents for cross-linking of proteins.

I. Introduction

Covalent cross-linking of proteins to other proteins, membranes, and smaller biomolecules is used widely in enzymology, structural biochemistry, biophysics, and chemical biology.[1-5] Numerous bifunctional organic reagents have been developed for this purpose and their properties and applications cataloged.[6] To be useful for cross-linking, a reagent should be soluble in water, selective toward particular amino acid side chains, and reactive at mild, preferably physiological, conditions. The cross-linked aggregate should be stable and yet cleavable, so that the original partners can be recovered from it. Most of the commonly used reagents are selective toward amino and sulfhydryl groups; others, such as photogenerated nitrenes, are nonselective.

This study introduces an inorganic approach to cross-linking of proteins. Various spectroscopic and chemical properties of transition metals render their complexes uniquely suited for specific binding to biological macromolecules, the ultimate multifunctional ligands.[7,8] Strong, easily detectable charge-transfer absorption bands of metal complexes are sensitive to the ligands and to the more distant environment; paramagnetic ions can serve as EPR probes and NMR relaxation agents; electron density renders the metals useful as tags for X-ray crystallography and electron microscopy; affinity toward ligands can be controlled by the oxidation state, by the hardness or softness of the metal atom, and by the structure and charge of the complex as a whole. Properties of platinum make it especially suitable for use in labeling reagents: it forms a myriad of stable complexes, undergoes various substitution reactions at convenient rates, and possesses an abundant and receptive isotope, ^{195}Pt, whose NMR chemical shifts span some 15 000 ppm.[9-11] A recent study from this laboratory has demonstrated the fitness of platinum for protein labeling.[12] The complex chloro(2,2′:6′,2″-terpyridine)platinum(II), $Pt(trpy)Cl^+$, proved to be a sensitive spectroscopic tag for cytochrome *c*. Its specificity (at pH 5) toward the imidazole side chain of histidine is caused by an interesting interplay between the steric and electronic effects of the aromatic terpyridine ligand.

Whereas the tridentate ancillary ligand ensures that only the Cl^- ion is displaced from $Pt(trpy)Cl^+$, and hence that only one protein molecule binds to the Pt atom, more than one Cl^- ion can, in principle, be displaced by proteins in the unidentate $PtCl_4^{2-}$ complex. Although $PtCl_4^{2-}$ has been used widely for protein tagging,[13-15] such cross-linking has not been observed because the reactions have commonly been carried out with crystals, in which the protein molecules are immobile. This report shows that proteins in solution, exemplified by cytochrome *c*, can be cross-linked selectively, under mild conditions, by the $PtCl_4^{2-}$ complex.

II. Materials and Methods

Chemicals. Imidazole (Im) and 2-fluoropyridine (2-Fpy) were obtained from Aldrich Chemical Co.; *N*-acetyl-L-methionine (AcMetH), Sephadex 75–50, aprotinin, trypsinogen inhibitor, trypsinogen, carbonic anhydrase, egg albumin, bovine albumin, and blue dextran were obtained from Sigma Chemical Co.; and *cis*-dichloro(2,2′-bipyridyl)platinum(II), $Pt(bpy)Cl_2$, was obtained from Strem Chemicals, Inc. K_2PtCl_4 was purchased from Aldrich and borrowed from Johnson Matthey, Inc. Distilled water was demineralized in a Barnstead Nanopure II apparatus.

Purification of Cytochrome *c*. Ferricytochrome *c* from horse heart (preparations of types III and VI) was obtained from Sigma Chemical Co. It was incubated with half an equivalent amount of $K_3Fe(CN)_6$ for several hours and the fully oxidized protein chromatographed, with 85 mM phosphate buffer at pH 7.0 as an eluent, on a column of CM 52 cation exchanger, obtained from Whatman.[16] Only the major fraction, containing ca. 85% of the commercial protein, was used in subsequent experiments. All dialyses were carried out in Amicon ultrafiltration cells with YM-5 membrane, whose threshold is 5 kDa, at 4 °C and under 50-atm pressure of purified nitrogen.

Determination of Molecular Mass. Size-exclusion gel chromatography was carried out on a column of Sephadex 75-50, sized 1.5 × 70 cm, at 4 °C. The eluent was 50 mM Tris-HCl buffer, 100 mM in KCl, at pH 7.5. The flow rate of 5.00 mL h^{-1} was maintained with an ISCO W1Z peristaltic pump. Absorbance was measured with an ISCO V-4 detector equipped with a strip-chart recorder. The following proteins, whose molecular weights in kilodaltons are given in parentheses, were used as standards: aprotinin (6.5), horse heart cytochrome *c* (12.5), trypsinogen inhibitor (20.1), trypsinogen (24.0), carbonic anhydrase (29.0), egg albumin (45.0), and bovine albumin (65.0). Each calibration point represents an average of two measurements. The void time was determined with blue dextran, whose molecular mass of 2 MDa falls far outside the working range of the gel, 5–66 kDa. For greater accuracy, elution times at constant flow rate, rather than elution volumes, were measured. The column was calibrated before every determination of the unknown molecular mass.

Absorption Spectroscopy. Electronic spectra were recorded with an IBM 9430 UV–vis spectrophotometer, equipped with a two-grating monochromator. Vibration spectra in the far-IR region were recorded

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with an IBM IR98 FT instrument, whose sample chamber was flushed with purified nitrogen. Nujol mulls were prepared from lyophilized proteins.

NMR Spectroscopy. The ^1H spectra at 300 MHz and ^{13}C spectra at 74.5 MHz were recorded with Nicolet NT 300 and Bruker WM 300 spectrometers using residual protons (in D_2O) and dioxane as respective internal standards. The protein-containing samples for ^1H NMR measurements were dialyzed into D_2O by ultrafiltration and then lyophilized with successive portions of D_2O to remove the NH resonances.[17-19] The ^{19}F spectra at 282 MHz were recorded with the Bruker WM 300 instrument. The linking reagents and model complexes were dissolved in water or in aqueous dimethylformamide (DMF), whereas the diprotein complex was dissolved in 150 mM phosphate buffer at pH 5.5. All samples contained 20% D_2O. A 10 mM solution of CF_3COOH in 85 mM phosphate buffer at pH 6.8, kept in a coaxial capillary tube, served as an external standard.

EPR Spectroscopy. The X-band spectra were recorded at 5 K with a Bruker ER200-SRC instrument equipped with an Oxford Instruments ESR900 cryostat. A double rectangular cavity had a nominal frequency of 9.56 GHz; modulation frequency was 100 kHz. The samples were fully oxidized: the native cytochrome *c* with $K_3Fe(CN)_6$; the modified one and the protein dimer with $[Co(phen)_3](ClO_4)_3$.[20] The oxidants were removed by dialysis into 85 mM phosphate buffer at pH 7.0.

Voltammetry. Differential-pulse and cyclic voltammograms were obtained with an IBM EC225 electrochemical analyzer equipped with a Houston Instruments Omnigraph 200 XY recorder. A BAS cell assembly consisted of an Ag/AgCl couple as reference, a Pt wire as auxiliary, and a 1.6-mm Au disk as working electrode. The composition of the sample solutions was as follows: ca. 0.5 mM in cytochrome *c* and 10 mM in 4,4′-bipyridyl (from Sigma Chemical Co.) as a mediator,[21] dissolved in 85 mM phosphate buffer at pH 7.0. A small, jacketed cell of 5 mL allowed experiments with 2-mL samples. The solutions were deoxygenated by gentle bubbling of argon and the blanket of this gas maintained during the measurements.

III. Preparation of Linking Reagents and of Model Complexes

***cis*- and *trans*-[Pt(2-Fpy)$_2$Cl$_2$].** The procedure was analogous to the published one,[22] except that 2-fluoropyridine was used instead of pyridine. To 100 mg (0.24 mmol) of K_2PtCl_4 in 2 mL of water was added 47 mg (0.48 mmol) of 2-Fpy. A precipitate, formed over several hours, was washed with cold water. The ^{19}F NMR spectrum of the solid dissolved in DMF showed it to consist of more than 95% of the cis isomer and of a very small amount of the trans isomer, exactly as reported originally.[22] The subsequent treatment of $[Pt(2\text{-}Fpy)_4]^{2+}$ with HCl yielded not the pure *trans*-$[Pt(2\text{-}Fpy)_2Cl_2]$, as was the case with the pyridine ligand, but a mixture of *cis*- and *trans*-$[Pt(2\text{-}Fpy)_2Cl_2]$. Since only the trans isomer proved soluble in benzene, it was extracted from the mixture with this solvent.

***cis*- and *trans*-[Pt(2-Fpy)$_2$(DMF)$_2$].** These linking reagents, containing labile DMF ligands, were prepared by addition of 6.8 mg (40 μmol) of $AgNO_3$ to 9.2 mg (20 μmol) of the mixture of *cis*- and *trans*-$[Pt(2\text{-}Fpy)_2Cl_2]$ dissolved in 1 mL of DMF. After the mixture was allowed to stand in the dark for 1 day, the precipitated AgCl was removed by centrifugation. The DMF complexes remained in clear solution.

***cis*-[Pt(bpy)(DMF)$_2$]$^{2+}$.** A solution of 8.4 mg (20 μmol) of *cis*-$[Pt(bpy)Cl_2]$ in 1 mL of DMF was treated with 6.8 mg (40 μmol) of $AgNO_3$, as described above. After the removal of AgCl by centrifugation, a clear solution of *cis*-$[Pt(bpy)(DMF)_2]^{2+}$ remained. Aliquots of this solution were used for incubation with cytochrome *c*.

Synthesis of [PtCl$_2$(AcMetH)$_2$]. A solution of 191 mg (1.0 mmol) of *N*-acetyl-L-methionine (AcMetH) in 4 mL of water was added dropwise, with stirring, to a solution of 208 mg (0.5 mmol) of K_2PtCl_4 in 2 mL of water. This reaction, and all other involving K_2PtCl_4, was carried out in the dark in order to minimize formation of Pt metal and other side reactions; the use of dilute (ca. 0.25 M) HCl instead of pure water as a solvent also contributed to the suppression of side reactions. Alternatively, AcMetH was added to an equimolar amount of preformed K-$[Pt(AcMetH)Cl_3]$.[23] After several hours at 50°, or after 2 days at room temperature, the solution of $[PtCl_2(AcMetH)_2]$ attained its equilibrium composition, as shown by ^1H NMR spectroscopy. Ionic species were removed by passing the solution through a column containing several grams of Dowex 50W-X8 cation exchanger in the H^+ form and then through a column containing several grams of Amberlite IRA-400 anion exchanger in the OH^- form. In either case the molar excess of the exchanger over the platinum complex was approximately 100-fold, to ensure complete retention of salts. The samples for NMR measurements usually were prepared with D_2O solvent; those prepared with H_2O were alternately desiccated and dissolved in D_2O before the measurements. The samples prepared in these two ways yielded identical spectra.

Model Complexes *trans*-[Pt(2-Fpy)$_2$L$_2$]$^{n+}$ with Imidazole (n = 2) and *N*-Acetylmethionine (n = 0) as L. The syntheses were carried out by a modified method of Pearson et al.[24] To 9.2 mg (20 μmol) of *trans*-$[Pt(2\text{-}Fpy)_2Cl_2]$ in 1 mL of solvent were added 6.8 mg (40 μmol) of $AgNO_3$ and 40 μmol of ligand L (2.7 mg of Im or 7.6 mg of AcMetH). The solvents used with Im and AcMetH were methanol and water, respectively. The reaction mixture was stirred, in the dark, for 5 days; during the first day it was also heated at 60 °C. After the AgCl precipitate was removed by centrifugation, the ^{19}F NMR spectra of the resulting solutions (see section VI) confirmed that the model complexes were formed completely.

IV. Preparation of Diprotein Complexes

***trans*-[PtCl$_2$(cyt)$_2$].** Ferricytochrome *c* was incubated, in the dark, with K_2PtCl_4 in 150 mM phosphate buffer at pH 5.5. The protein concentration was 2 mM, usually 25 mg (2 μmol) in 1 mL of the buffer. The concentration of the linking reagent was varied systematically from 1 to 12 mM, i.e., from 0.42 to 5.0 mg (1 to 12 μmol) in 1 mL. The optimal yield of the diprotein complex, 15%, was achieved with equimolar amounts of the protein and $PtCl_4^{2-}$ in 1-day incubation. The unconsumed K_2PtCl_4 was removed by dialysis into 150 mM phosphate buffer at pH 5.5. The protein solution was then subjected to cation-exchange chromatography on a CM 52 column sized 1.5 × 25 cm with the same 150 mM buffer as an eluent, or to size-exclusion chromatography, as described in section II. The first exploratory experiments were carried out with ferrocytochrome *c*. The protein was treated with 1.5 times the equivalent amount of ascorbic acid, dialyzed, and incubated with K_2PtCl_4 under the atmosphere of nitrogen. Since ferricytochrome *c* proved entirely stable under the reaction conditions, the main work was done with it.

Cation-exchange chromatography yielded three well-separated bands. The native cytochrome *c* and its derivative labeled with $PtCl_3^-$ were eluted with the 150 mM buffer. The third band remained immobile atop the column, but was eluted easily when the buffer was made 100 mM in NaCl.

Samples for size-exclusion chromatography were dialyzed into the Tris-HCl buffer. The raw product of incubation, a mixture of protein derivatives, yielded two fractions. The first one, corresponding to a molecular mass of 30.7 ± 0.8 kDa, contains the diprotein complex, i.e., the protein dimer. The second band, with molecular mass of 13.0 kDa, evidently consists of monomeric cytochrome *c*. Size-exclusion chromatography of the separate fractions from the CM 52 column confirmed that the two mobile bands contained monomeric cytochrome *c* and that the third one contained the protein dimer.

***trans*-[Pt(2-Fpy)$_2$(cyt)$_2$].** The reagent *trans*-$[Pt(2\text{-}Fpy)_2(DMF)_2]$ was used in a procedure analogous to the one described above for $PtCl_4^{2-}$. The solution of the linking reagent in DMF was concentrated so that the required amount of it was contained in an 80-μL aliquot. Since the volume of the aqueous (buffered) protein solution was 2 mL, the resulting reaction mixture contained only 4% DMF. Higher concentrations of DMF were avoided lest cytochrome *c* become denatured.

Attempted Preparation of *cis*-[Pt(bpy)(cyt)$_2$]. Incubation of cytochrome *c* with *cis*-$[Pt(bpy)(DMF)_2]$ was carried out as with *trans*-$[Pt(2\text{-}Fpy)_2(DMF)_2]$. Cation-exchange chromatography on a CM 52 column yielded three major and two minor fractions. The first major one exhibits the UV–vis spectrum of the native protein. The $Pt(bpy)^{2+}$ chromophores in the other four fractions were easily detected and quantitated on the basis of their characteristic absorption maximum at 320 nm and of a shoulder at ca. 308 nm. The second and third major fractions each contain one platinum tag per protein, whereas the two minor fractions consist of multiply labeled protein derivatives.[25] Size-exclusion chromatography of the reaction mixture not subjected to cation-exchange chromatography yielded a single band, corresponding to the molecular mass of cytochrome *c*. Evidently, all the fractions separated on the CM 52 column consist of monomers—of cytochrome *c* and of its

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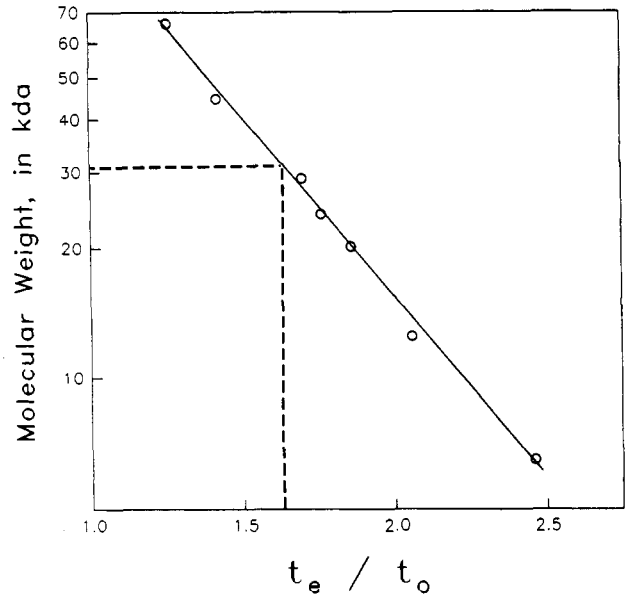


Figure 1. Size-exclusion chromatography of *trans*-$[PtCl_2(cyt)_2]$, a complex containing cytochrome *c* from horse heart. The molecular mass of 30.7 ± 0.8 kDa is determined by the procedure described in section II. Elution and void times, designated t_e and t_0, respectively, are measured more accurately than the corresponding volumes.

labeled derivatives. Incubation of singly labeled protein derivatives, which may be formulated *cis*-[Pt(bpy)(cyt)L], wherein L is DMF or H_2O, with an equivalent amount of the native cytochrome *c* also failed to produce any diprotein complexes. Size-exclusion chromatography showed the reaction mixture to consist solely of monomeric protein species.

Cleavage of Diprotein Complexes. Complex $[PtCl_2(cyt)_2]$ was incubated with an excess of thiourea in phosphate buffer for 2 days. Size-exclusion chromatography showed that the reaction mixture contained monomeric cytochrome *c*, i.e., that the Pt–protein bonds had been cleaved.

V. Formation and Structure of the Diprotein Complexes

Linking Reactions and the Molecular Mass. The reactions between equimolar amounts of cytochrome *c* and complexes $PtCl_4^{2-}$, *trans*-$[Pt(2\text{-}Fpy)_2(DMF)_2]$, *cis*-$[Pt(bpy)(DMF)_2]$, and *cis*-[Pt(bpy)(cyt)L] take place under mild conditions, namely at 4 °C or at room temperture. The yield of the diprotein complex $[PtCl_2(cyt)_2]$ is 15%. Incubation for 1 day is sufficient; the yield is not improved after 3 months at room temperature. Ferricytochrome *c* proved entirely stable under the reaction conditions, so the ferrous form of the protein, which has a tighter conformation,[26] did not need to be used.

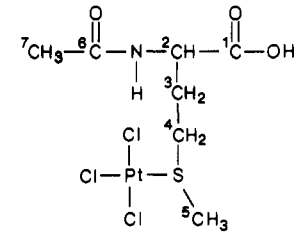
The first two bands obtained by cation-exchange chromatography of the mixture of cytochrome *c* and $PtCl_4^{2-}$ contain native cytochrome *c* and its labeled derivative, $[PtCl_3(cyt)]$. The third band, which adhered to the top of the column, was eluted easily when NaCl was added to the buffer. This chromatographic behavior is identical with that of the cytochrome *c* from baker's yeast, a protein that contains free cysteine 102 and dimerizes by the formation of a disulfide bond.[27,28] This great similarity in the chromatographic mobility was an early indication that the third fraction obtained from the cation-exchange column contains a diprotein complex.

The molecular mass of 30.7 ± 0.8 kDa, determined by size-exclusion chromatography as shown in Figure 1, confirmed this hypothesis. The value is greater than that expected of $[PtCl_2(cyt)_2]$ by about 18%, but this discrepancy becomes understandable in view of the mechanism of gel-filtration chromatography. A strict proportionality between the elution rate and the log m_r (itself an approximate measure of the biopolymer size) obtains only with protein molecules of similar shapes. The seven globular, one-chain proteins that are used as standards indeed fall on a straight line, as Figure 1 shows. Linking of two spheroidal molecules of cytochrome *c* via a $PtCl_2$ bridge, however, results in an elongated diprotein complex, which, to the Sephadex gel, appears a little larger than a spheroidal protein of the same molecular mass.

Table I. ^{13}C NMR Chemical Shifts (ppm Downfield from Me_4Si)

atom[a]	free AcMetH[b]	$[PtCl_3(AcMetH)]^-$[c]	$[PtCl_2(AcMetH)_2]$
C^5	14.1	20.8	20.8 (cis) 19.2 (trans)
C^7	21.7	22.2	22.0
C^3	29.4	28.5	28.5
C^4	29.9	34.8	34.9 (cis) 35.1 (trans)
C^2	51.7	51.7	51.4
C^6	174.2	174.3	173.9
C^1	175.4	174.6	174.0

[a] Atom labeling is shown below:



[b] *N*-Acetyl-L-methionine. [c] From ref 23.

The Binding Sites. The amino acid side chains through which the protein molecules coordinate to the Pt atom are identified on the basis of unambiguous crystallographic and NMR findings by others. Dickerson and co-workers introduced, and many other applied, $PtCl_4^{2-}$ as a specific reagent for the methionine residue in proteins.[13-15] It labels Met 65 in ferricytochrome *c* from horse virtually exclusively although incubation at pH 6.2 resulted in very slight additional labeling of His 33.[13] Studies by ^1H NMR spectroscopy showed that, at pH 5.5, $PtCl_4^{2-}$ binds exclusively to methionine: to Met 29 in ribonuclease[29] and, again, to Met 65 in cytochrome *c* from tuna.[30] We carried out the incubation at pH 5.5, so that the imidazole side chain, whose pK_a is 6.4,[31] is protonated and unreactive. Labeling of Met 80, an axial ligand to the Fe atom in heme, is ruled out on the basis of the aforementioned crystallographic and NMR studies and because the spectroscopic and redox properties of the cytochrome *c* are not altered in the diprotein complexes. (See section VI.) To conclude, the proteins are coordinated to the Pt atom through the S atoms in their respective Met 65 residues.

The new protein dimers are remarkably stable owing to the well-known affinity of thioether ligands for the Pt(II) atom. The $PtCl_2(cyt)_2$ complex, for example, remains intact even after several months in the buffered solution at room temperature. Size-exclusion chromatography of the aged solution indicates a complete absence of the monomeric cytochrome *c*, i.e., of dissociation. The covalent bonds between the Pt atom and its binding sites in the two proteins can be cleaved, however, by highly nucleophilic ligands. Thiourea, designated tu, can displace the proteins and extrude the platinum link, as shown in eq 1. This combination

$$[PtCl_2(cyt)_2] + 4tu \rightarrow 2cyt + [Pt(tu)_4]Cl_2 \qquad (1)$$

of stability under ordinary conditions and easy removability under conditions harmless to the protein is a particularly useful feature of the PtL_2 links.

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Table II. Electronic and Structural Characteristics of Native Cytochrome *c* from Horse Heart and of Its Two Bis(protein)platinum(II) Complexes

property		cyt	*trans*-$[PtCl_2(cyt)_2]$	*trans*-$[Pt(2\text{-}Fpy)_2(cyt)_2]$
EPR *g* values[a]	g_x	1.26	1.3	1.3
	g_y[b]	2.339	2.291	2.299
	g_z	3.018	2.976	2.986
	$g_\parallel$	2.167[c]	1.991[d]	1.989[d]
	$g_\perp$	6.045[c]	5.948[d]	5.893[d]
UV–vis abs max, nm[e]		409	410	410
		535	535	534
		695	695	695
reduction pot., mV vs. NHE[f]		257 ± 5	220 ± 5	
hyperfine-shifted ^1H NMR resonances of CH_3 groups, ppm[g]	CH_3 in ring IV	35.68	35.7	
	CH_3 in ring II	32.53	32.53	
	S–C*H*–CH_3 in ring II or CH 2 and CH 4 of His 18	24.5	25.4	
	β-CH_2 of propionate in ring IV	19.24	19.3	
	β-CH_2 of His 18	14.63	14.65	
	unassigned	12.71	12.8	
	β-CH_2 of propionate in ring IV	11.41	11.4	
	CH_3 of Met 80	−24.49	−24.49	
	γ-CH_2 of Met 80	−28.3	−28.3	
far-IR bands, cm^{-1}		345[h]	343	

[a] In 150 mM phosphate buffer at pH 7.0. [b] Measured at the peak maximum. [c] High-spin ferriheme in less than 2% of the native protein. [d] High-spin ferriheme in less than 10% of the diprotein complex. [e] In 85 mM phosphate buffer at pH 5.5. [f] In 85 mM phosphate buffer at pH 7.0 and at 25 °C, with 4,4′-bipyridyl as mediator. [g] With respect to DSS as standard. [h] Mixture of native cytochrome *c* and K_2PtCl_4 in the molar ratio of 2:1.

Model Complexes and Configuration of the Diprotein Complexes. The model complex of *N*-acetylmethionine, $[PtCl_2(AcMetH)_2]$, is obtained in substitution reactions shown in eq 2, which are

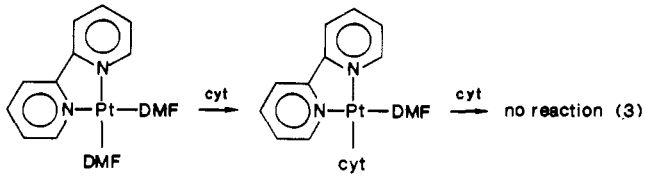
$$PtCl_4^{2-} + AcMetH \xrightarrow[-Cl^-]{} [PtCl_3(AcMetH)]^- \xrightarrow[AcMetH]{-Cl^-} [PtCl_2(AcMetH)_2] \quad (2)$$

$$PtCl_4^{2-} + 2AcMetH \xrightarrow[-2Cl^-]{} [PtCl_2(AcMetH)_2]$$

cis:trans = 4:1

followed easily by the changes in the ^{13}C and ^1H NMR spectra of the ligand.[23] As Table I shows, only the signals due to atoms C^5 and C^4 move significantly upon coordination. Since these atoms are adjacent to the S atom, AcMetH is bonded to the Pt atom as a monodentate thioether ligand. With its amino group protected and the carboxyl group virtually unreactive toward Pt(II),[9-11] the AcMetH ligand mimics a Met residue in the protein. The ^{13}C NMR signals of C^5 and C^4 are split into two peaks each, whose relative intensities are 4:1. The more abundant isomer evidently resembles the monosubstituted complex, $[PtCl_3(AcMetH)]^-$, in having the thioether and Cl^- ligands trans to ech other, i.e., the cis configuration. The less abundant isomer then has the trans configuration. The isomerization is monitored in the ^1H NMR spectra, which are recorded much faster than the ^{13}C spectra. Because of the kinetic trans effect of the first AcMetH ligand, the first isomer of $[PtCl_2(AcMetH)_2]$ to form in either of the routes shown in eq 1 is the trans isomer, having the CH_3S signal at 2.33 ppm. Within 1 h the cis isomer, having the CH_3S signal at 2.46 ppm, becomes the more abundant one. The ratio at equilibrium becomes 4:1, exactly as found by ^{13}C NMR spectroscopy.

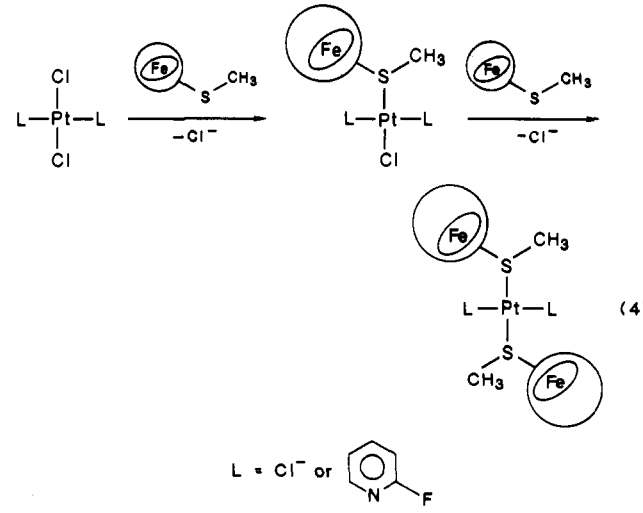
These stereochemical studies of the model complex $[PtCl_2(AcMetH)_2]$ posed the question of the configuration at the Pt atom in the diprotein complexes, $[PtL_2(cyt)_2]$. It was addressed by experiments in which cytochrome *c* was incubated with *cis*-[Pt(bpy)(DMF)$_2$], a compound whose disubstituted derivative would have to adopt a cis configuration. As eq 3 shows, only monoprotein



complexes are obtained. The lability of DMF ligands notwithstanding, the isolated monoprotein complex cannot bind the second molecule of cytochrome *c*. Since two cytochrome molecules apparently cannot occupy cis positions, the diprotein complexes probably have trans configurations, as would be expected in view of the protein size.

The shortest interprotein distance in the *trans*-$[PtL_2(cyt)_2]$ complex can be estimated at 4.6 Å, twice the length of a typical Pt–SR_2 bond.[32,33] The shortest interatomic contacts in the crystal between the two molecules of tuna cytochrome *c* in the same unit cell fall in the range 4.5–5.0 Å.[34] Although inexact, this comparison indicates that trans linking through a PtL_2 fragment does not bring the protein molecules prohibitively close to each other. Coordination in the cis positions is ruled out, for it would place the S donor atoms about 3.2 Å from each other.

Formation of the bis(cytochrome) complexes is shown schematically in eq 4. The first protein ligand, bonded to the Pt atom



through the S atom of Met 65, facilitates the second substitution by a kinetic trans effect, a well-known property of thioether ligands.

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Table III. ^{19}F NMR Spectra of Model Complexes and of a Bis(cytochrome *c*)platinum(II) Complex

	solvent[a]	^{19}F chem shift, ppm from CF_3COOH	$^3J(^{195}Pt-^{19}F)$, Hz
cis-$[Pt(2\text{-}Fpy)_2Cl_2]$	DMF	13.14	174
	H_2O	14.10	165
trans-$[Pt(2\text{-}Fpy)_2Cl_2]$	DMF	15.58	184
	H_2O	15.91	160
trans-$[Pt(2\text{-}Fpy)_2(Im)_2]$[b]	H_2O	13.38	214
trans-$[Pt(2\text{-}Fpy)_2(AcMetH)_2]$[c]	H_2O	12.61	172
trans-$[Pt(2\text{-}Fpy)_2(cyt^{II})_2]$[d]	H_2O	14.7	
trans-$[Pt(2\text{-}Fpy)_2(cyt^{III})_2]$[d]	H_2O	14.7	

[a] 80% liquid indicated and 20% D_2O. [b] Im is imidazole. [c] AcMetH is *N*-acetyl-L-methionine. [d] The oxidation state of iron is indicated.

VI. Structural and Redox Properties of the Diprotein Complexes

The diprotein complexes *trans*-$[PtCl_2(cyt)_2]$ and *trans*-$[Pt(2\text{-}Fpy)_2(cyt)_2]$ were compared with one another and with the native cytochrome *c* by various physical methods in order to determine whether linking alters the protein structure and properties of its active site. Most of the findings are presented in Table II.

The EPR spectra confirm that the electronic structure of the ferriheme is virtually unperturbed upon linking of the proteins; the slight differences among the *g* values fall within the error limits of the measurement.[35-37] A small amount (less than ca. 10%) of the protein exists in the high-spin Fe(III) form, characterized by *g* values of about 2.0 and 6.0. Since the UV–vis absorption maxima are not moved significantly, whereas the Soret and other bands are known to move to shorter wavelengths in high-spin heme proteins,[38] this form must represent a very minor constituent of the protein dimers.

Particularly diagnostic is the absorption band at 695 nm, whose intensity depends in part on the interactions between the Fe atom and its axial ligands, Met 80 and His 18.[39-41] Although its absorptivity of ca. 1000 M^{-1} cm^{-1} is too low to permit accurate comparisons of the band intensity among proteins, the presence of this band in the spectra of the dimers indicates that the Fe atoms remain hexacoordinate. Dissociation of the Met 80 ligand, a process detectable even in the native cytochrome *c*[42] and responsible for conversion to the high-spin form, is slightly enhanced in the protein dimer.

The reduction potential of the diprotein complex, ca. 220 mV, is lower by ca. 30 mV than that of the native protein because of the presence of some high-spin form. Since the reduction potential of the high-spin cytochrome *c* from *Rhodopseudomonas rubrum* is 10 mV at pH 7,[43] 240 mV below that of the low-spin proteins, the observed difference of 30 mV can be taken as further evidence that only a minor fraction of the diprotein complex exists in the high-spin form.

The proton NMR spectrum of the active site in heme proteins depends markedly on the interactions between the paramagnetic Fe(III) atom and its axial ligands and between the heme periphery and the side chains of neighboring amino acid residues.[44-49] Particularly sensitive to the protein conformation are the hyperfine ^1H shifts, which have been assigned.[50-54] They remain unperturbed in the diprotein complex.

The $PtCl_2$ link between the protein molecules is directly detectable by IR spectroscopy. The complex *trans*-$[PtCl_2(cyt)_2]$ exhibits a single band in the region 650–200 cm^{-1}, as expected of a *trans*-$PtCl_2$ fragment. The position of this Pt–Cl stretching band is virtually the same as that of the band exhibited by a mixture of cytochrome *c* and K_2PtCl_4 in the ratio of 2:1.

The presence of ancillary 2-fluoropyridine ligands permits easier detection of the link by ^{19}F NMR spectroscopy. As Table III shows, the ^{19}F chemical shift and the $^{195}Pt-^{19}F$ coupling constant in model complexes depend on the other two ligands coordinated to the Pt atom. The ^{19}F chemical shift of *trans*-$[Pt(2\text{-}Fpy)_2(cyt)_2]$ falls in the expected interval, but the width of the resonance band, caused by the slow nuclear relaxation in the macromolecular complex, limits the usefulness of ^{19}F NMR spectroscopy for structural studies. Since direct methods are preferable, the binding site on the protein was identified unambiguously on the basis of the known selectivity of the $PtCl_4^{2-}$ complex toward cytochrome *c*, as explained in section V.

VII. Advantages and Prospective Applications of Cross-Linking via Platinum Complexes

This report introduces inorganic complexes as reagents for cross-linking of proteins. In addition to the general properties of transition metals, mentioned in section I, platinum exhibits several particular advantages. Whereas the common organic agents react with amino and thiol groups, $PtCl_4^{2-}$ seems to be specific for thioether groups. The reagent is stable in aqueous solution and reacts with proteins under physiological conditions. Behavior of the prototypal bis(cytochrome *c*) complexes indicates that the cross-links are stable indefinitely under ordinary conditions and yet cleavable in a mild reaction. Especially attractive is the prospect of altering the reagent properties by varying the ancillary ligands; in this study they are Cl^- and 2-Fpy. Bimetallic complexes in which the metal atoms are tethered with cyclic and acyclic ligands of different length promise to combine the advantages of the existing organic and new inorganic reagents. Our research in these directions continues.

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