$[Fe(C_5H_5)_2]^{\bullet+}[C_4(CN)_6]^{\bullet-}$. In contrast the magnetic susceptibility of $[Fe(C_5Me_5)_2]^{\bullet+}[C_4(CN)_6]^{\bullet-}$ does not show evidence for ferromagnetic coupling. The susceptibility obeys the Curie–Weiss expression with $\theta = -3.4$ K and the effective moment, μ_{eff}, is 3.34 μ_B[35] (Figure 9). The moment vs. temperature variation is consistent with depopulation of the spin–orbit levels in a paramagnet and/or weak antiferromagnetic coupling. Similar behavior has also been reported for $[Fe(C_5H_5)_2]^{\bullet+}$ salts with diamagnetic anions, e.g., $[I]_3^-$ ($\theta = -1.33$ K)[36] and $[DDQ]^-$ ($\theta = -4.23$ K).[4b,32] Additionally, the susceptibility is essentially field independent and the low-temperature susceptibility plotted as χ^{-1} vs. T does not have an upturn to a constant value of χ^{-1} as noted for ferromagnetic $[Fe(C_5Me_5)_2]^{\bullet+}[C_4(CN)_6]^{\bullet-}$ and $[Fe(C_5Me_5)_2]^{\bullet+}$-$[TCNE]^{\bullet-}$.[5,28]

Conclusion

We have shown that $[Fe(C_5Me_5)_2]^{\bullet+}[C_4(CN)_6]^{\bullet-}$ like $[Fe(C_5Me_5)_2]^{\bullet+}[TCNE]^{\bullet-}$[5,28] possesses a 3-D ferromagnetic ground state. Both of these ferromagnetic charge-transfer complexes possess the same $\cdots D^{\bullet+}A^{\bullet-}D^{\bullet+}A^{\bullet-}\cdots$ structural motif; however other similarly structured complexes exhibit different magnetic behaviors, i.e., para- and metamagnetism. It seems clear that further studies are in order before we fully understand the specific structural/electronic features leading to a particular magnetic ground state in this class of compounds. This is particularly the case as substitution of $[Fe(C_5H_5)_2]^{\bullet+}$ for $[Fe(C_kMe_5)_2]^{\bullet+}$ although possessing the same electronic structure exhibits paramagnetic behavior. We attribute this to the latter salt belonging to a different structure type; however, crystals suitable for X-ray analysis have yet to be prepared. The structural determination of $[Fe(C_5H_5)_2]^{\bullet+}[C_4(CN)_6]^{\bullet-}$ should expand our knowledge of the structure–function relationship in this class of compounds.

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Supplementary Material Available: Tables of the observed and calculated structure factors for $[Fe(C_5Me_5)_2]^{\bullet+}[C_4(CN)_6]^{\bullet-}$ (5 pages). Ordering information is given on any current masthead page.

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A Transition-Metal Chromophore as a New, Sensitive Spectroscopic Tag for Proteins. Selective Covalent Labeling of Histidine Residues in Cytochromes *c* with Chloro(2,2′:6′,2″-terpyridine)platinum(II) Chloride

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Abstract: Reactivity and selectivity of $Pt(trpy)Cl^+$ toward proteins are studied with cytochromes *c* from horse and tuna as examples. The new transition-metal reagent is specific for histidine residues at pH 5. The reaction, facile one-step displacement of the Cl^- ligand by imidazole, produces good yield. The binding sites, His 26 and His 33 in the horse protein and His 26 in the tuna protein, are identified by UV–vis spectrophotometry and by peptide-mapping experiments. Model complexes with imidazole, histidine, histidine derivatives, and histidine-containing peptides are prepared and characterized. The covalently attached $Pt(trpy)^{2+}$ labels allow easy separation of the protein derivatives by cation-exchange chromatography. The labels do not perturb the conformation and reduction potential of cytochrome *c*, as shown by UV–vis spectrophotometry, cyclic voltammetry, differential-pulse voltammetry, EPR spectroscopy, and ^1H NMR spectroscopy. The selectivity of $Pt(trpy)Cl^+$ is entirely opposite from that of $PtCl_4^{2-}$ although both of them are platinum(II)–chloro complexes. Owing to an interplay between the steric and electronic effects of the terpyridyl ligand, the new reagent is unreactive toward methionine (a thio ether) and cystine (a disulfide), which are otherwise highly nucleophilic ligands, but very reactive toward imidazole, which is otherwise a relatively weak ligand. Unusual and useful selectivity of preformed transition-metal complexes toward proteins evidently can be achieved by a judicious choice of ancillary ligands. The strong UV and visible bands, some of them unobscured by the protein absorption, render the $Pt(trpy)^{2+}$ chromophore easy to detect and quantitate. The difference between the spectra of the tags bonded to His 26 and His 33 residues in the horse protein shows that the chromophore is sensitive not only to the nature of the binding site but also to its environment.

I. Introduction

Covalent modification of amino acid side chains has proved useful in studies of enzymes and proteins in general.[1-3] After many organic reagents have been developed, the promise of inorganic ones is becoming recognized. Various properties of transition metals render their complexes uniquely suited for labeling of biological macromolecules.[4,5] Charge-transfer absorption bands are strong and sensitive to the environment; paramagnetic ions serve as EPR probes and NMR relaxation agents; heavy metals are used as tags for X-ray crystallography and electron microscopy; binding selectivity can be controlled by the oxidation state, hardness or softness, coordination number, and charge.

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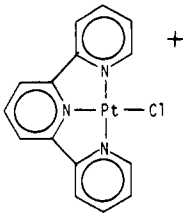
Platinum has particular advantages: it forms stable complexes, reacts at convenient rates, and possesses an abundant and receptive isotope, ^{195}Pt, whose chemical shifts span some 15 000 ppm.[6-8] Although $PtCl_4^{2-}$, as well as some other heavy-atom compounds, has proven extremely useful in protein crystallography,[9-11] few inorganic labeling reagents have been designed intentionally and deliberately. In this report chloro(terpyridine)platinum(II), Pt(trpy)Cl^+, is introduced as a new reagent for covalent modification of proteins; this compound, shown below, had previously been used as an intercalator for DNA.[12,13]



Selectivity toward functional groups or sites in a protein depends on the coordination affinity of metals toward them. In one method, a side chain is converted into a chelating agent and a metal ion introduced into it.[14-20] In another, a substitution reaction is effected between a protein and a preformed labile metal complex.[21-28] Our approach involves the preformed reagent, Pt(trpy)Cl^+, whose three main advantages, as demonstrated in this study, are the following. (1) It binds in a one-step displacement of the Cl^- ligand, with a good yield. The mild conditions and absence of other reagents make side reactions unlikely. (2) Its selectivity is opposite from that of the commonly used $PtCl_4^{2-}$. (3) The direct attachment of the Pt(trpy)$^{2+}$ chromophore to the protein enhances its value as a probe or a tag. Each of these three characteristics is influenced by the terpyridine ligand as follows. (1) By chelating the Pt atom it permits only substitution of Cl^-. (2) By an interplay of steric and electronic effects, it accelerates the substitution reaction with histidine (His) but prevents reaction with methionine (Met) and disulfide. (3) It introduces very strong absorption bands in the visible and near-UV regions. Whereas none of the common organic reagents seem to be specific for the histidine side chain,[29,30] the new reagent modifies it selectively at pH 5, under very mild conditions.

II. Experimental Section

Materials and Methods. The proteins, trypsin treated with TPCK, amino acids, and peptides were obtained from Sigma Chemical Co. Horse-heart cytochrome *c* (of type VI) was oxidized with $K_3Fe(CN)_6$ and purified by chromatography on CM 52 cellulose.[31] Tuna-heart cytochrome *c* (of type XI) was used as received. Chloro(2,2′:6′,2″terpyridine)platinum(II) chloride dihydrate, [Pt(trpy)Cl]$Cl\cdot 2H_2O$, was purchased from Strem Chemicals.[32] Compound $[Co(phen)_3](ClO_4)_3$ was prepared readily.[33] Deuteriated chemicals were from Aldrich Chemicals. Ultrafiltration was performed with Amicon YM-5 membranes under nitrogen at 4 °C. Microanalysis was done by Galbraith Laboratories.

The ^1H NMR spectra were recorded in D_2O solutions with Nicolet NT 300 and Bruker WM 300 spectrometers. The proteins were dialyzed into D_2O by ultrafiltration and then lyophilized repeatedly with D_2O. The NH resonances were removed,[34-36] and resolution was enhanced,[37] by standard techniques. The ^{195}Pt NMR spectra of 15–30 mM solutions of Pt(trpy)L^{2+} complexes were recorded with the Bruker instrument at 64.4 MHz, using a 20-mm broad-band probe. The chemical shifts are expressed with respect to $PtCl_4^{2-}$.[38,39] Absorption spectra of 10 μM solutions, which obey Beer's law,[32] were recorded with an IBM 9430 UV–vis spectrophotometer, equipped with a two-grating monochromator. The X-band EPR spectra at 6 K were obtained with a Bruker ER 200D instrument.

Peptide-mapping experiments (tryptic hydrolysis and separation of the resulting peptides by HPLC) followed published procedures[40,41] except that digestion was performed at pH 7.5 for 10 h. The absorbance at 220 nm is due to peptide bonds and that at 342 nm, to Pt(trpy)His^{2+} chromophore. The retention times of the Pt(trpy)L^{n+} complexes, wherein L is Cl^-, His, Im, His-Lys, Gly-His-Gly, and Ala-Pro-Gly-Asp-Arg-Ile-Tyr-Val-His-Pro-Phe, were measured under the conditions used for the tryptic peptides. Although the labels are otherwise stable in solution, they undergo partial dissociation during incubation with trypsin. Digestion of the labeled cytochrome *c* is less efficient (yields a smaller amount of peptides) than that of the native protein.

Differential-pulse, constant-potential differential-pulse, and cyclic voltammograms were obtained with an IBM EC 225 voltammetric analyzer and a BAS cell assembly according to a published procedure.[42] Concerned that the Pt labels might be displaced from the proteins by the mediator, 4,4′-dipyridyl, we dialyzed the samples after the electrochemical measurements and proved, by the UV–vis spectra, that labels remained.

The Fenske–Hall method for molecular-orbital calculations has been described elsewhere.[43] The dimensions of the Pt(trpy)$^{2+}$ fragment[13] and of the Pt–$S(CH_3)_2$[44,45] and platinum–imidazole[46,47] units were taken from

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actual structures. The standard basis sets were used.[48]

Protein Modification. Ferricytochrome *c* was incubated with an equimolar amount of [Pt(trpy)Cl]Cl, 2 mM each, at room temperature in 0.1 M acetate buffer at pH 5.0 for 24 h. Procedures involving 5.0 or 25 mg of the protein and 0.20 or 1.0 mg of the platinum complex proved particularly convenient although equal yields were obtained with both larger and smaller quantities. The reaction was terminated by ultrafiltration into the acetate buffer at pH 5.0 and subsequently into 85 mM phosphate buffer at pH 7.0. The products were separated on a CM 52 column, equilibrated with this phosphate buffer at 4 °C. About 1 mg of cytochrome was applied per 1 mL of the resin bed. The horse cytochrome yielded, in this elution order, native protein (N), 38%, and major singly-labeled (S_A), 42%; minor singly-labeled (S_B), 2%; and doubly-labeled (D), 7%, derivatives. The first three fractions were eluted with 85 mM phosphate buffer, whereas derivative D required a gradient to 125 mM buffer. The tuna cytochrome yielded two fractions, eluted in the same order: native (N), 90%, and derivative S_B, 7%. Incubation of either cytochrome with a tenfold molar excess of [Pt(trpy)Cl]Cl did not yield any new products. Before spectroscopic and electrochemical studies, the separated protein derivatives were oxidized with $[Co(phen)_3](ClO_4)_3$, and this complex was removed by ultrafiltration. Treatment of any modified cytochrome *c* with 5 equiv of thiourea, SCN^-, or I^- (designated L) removed the tags completely; the native protein and the corresponding $Pt(trpy)L^{n+}$ complex were separated by ultrafiltration.

Survey of Amino Acids. Every amino acid containing a heteroatom in the side chain was incubated with [Pt(trpy)Cl]Cl at room temperature for several days; the concentrations of both reactants were 5 mM. The following amino acids caused no change in the UV–vis spectrum under the general conditions of protein modification: Lys, Trp, Arg, Asp, Asn, Glu, Gln, Pro, Thr, Ser, Tyr, and Met. With cysteine (Cys), homocysteine, and reduced glutathione the color changed immediately upon mixing, as expected.[49] With histidine (His), the color change developed over an hour. Reaction with Arg, which occurs under different conditions, will be the subject of a separate publication.

Complexes $[Pt(trpy)L]^{2+}$ with Histidine and Its Homologs. A solution containing 15.5 mg (0.1 mmol) of free base L-histidine in 1.0 mL of water was added dropwise to a stirred solution containing 53.2 mg (0.1 mmol) of $[Pt(trpy)Cl]Cl\cdot 2H_2O$ in 4.0 mL of water, and the mixture was kept at 50 °C. Although the color soon changed from dark orange to pale yellow and the absorbance quotient (A_{342}/A_{328}) became constant in less than an hour, the heating was continued for 1 day. A concentrated aqueous solution containing 68.4 mg (0.2 mmol) of $NaBPh_4$ was added dropwise to the stirred reaction mixture. After cooling, the yellow precipitate was filtered off, washed with cold water, and dried overnight in air: yield was 82 mg or 65%. Anal. Calcd for $[Pt(trpy)His](BPh_4)_2\cdot 2H_2O$: C, 65.87; H, 5.13; N, 6.68. Found: C, 65.25; H, 4.91; N, 6.53.

Ligands imidazole (Im), N^{α}-acetyl-L-histidine (AcHis), L-histidyl-L-histidine (His-His), L-histidyl-L-lysine (His-Lys), and glycyl-L-histidylglycine (Gly-His-Gly) were similarly treated with [Pt(trpy)Cl]Cl. After the quotient A_{342}/A_{328} became constant, the complexes in solution were characterized by UV–vis and ^1H NMR spectroscopy. The Job's plot[49] showed that Gly-His-Gly and $[Pt(trpy)Cl]^+$ react in the ratio of 1:1. The spectrophotometrically determined overall rate constants, k_{obsd}, for the reaction between 20 μM [Pt(trpy)Cl]Cl and 0.40 mM imidazole at pH values of 5.5, 7.0, and 8.6 are, respectively, 5.5×10^{-5}, 8.6×10^{-5}, and 2.7×10^{-3} s^{-1} at 25 °C.

Unreactivity of Thio-Ether and Disulfide Ligands. Compound [Pt(trpy)Cl]Cl was treated with DL-methionine, *N*-acetyl-DL-methionine, *N*-acetyl-L-methionine amide, L-methionine methyl ester hydrochloride, *S*-methyl-L-cysteine, tetrapeptide Trp-Met-Asp-Phe hydrochloride, cystine, and oxidized glutathione. First, [Pt(trpy)Cl]Cl was incubated with an equimolar amount of each potential ligand at the concentrations of 0.5, 1, 2, 5, 10, or 20 mM, and the mixtures were heated at 50, 60, 80, 90, or 100 °C for 10 h. Next were incubations with tenfold excess of *N*-acetylmethionine for several hours at 90 °C and with 100-fold excess of methionine for 75 days at room temperature. Finally, compound [Pt(trpy)Cl]Cl was treated with $AgNO_3$, AgCl was removed, and an equivalent amount of the ligands was added. None of the experiments caused significant changes in the UV–vis and ^1H NMR spectra of the starting complex and of the added ligands. Cation-exchange chromatography of the mixtures permitted virtually full (ca. 95%) recovery of [Pt(trpy)Cl]Cl.

III. The Model Complexes and Reactions

Complexes of Histidine and Its Derivatives. Imidazole, histidine, and their homologs displace the Cl^- ligand from $Pt(trpy)Cl^+$, as

Table I. Spectroscopic Properties of $[Pt(trpy)L]^{n+}$ Complexes and of Free Imidazole-Containing Ligands L

L	*n*	absorptivity, ϵ,[a] M^{-1} cm^{-1}				$\epsilon_{342}/\epsilon_{328}$	^1H NMR chemical shift,[c] ppm											^{195}Pt NMR,[d] ppm
							Pt(trpy)			Im		aliphatic						
		342	328	270	242		$H^{4,4'}$	$H^{3,3'}$	H^6	H^5	H^{C2}	H^{C5}	H^7	H^8	H^9	$H^{10,11}$	H^{12}	H^{13}	
Cl^-	1	13 300		24 300	27 800		8.08, m, 3 H	7.88, d, 4 H	7.82, m, 2 H	7.37, m, 2 H									−1080
Im	2	16 000	10 400	21 200	31 800	1.54	8.33, m, 3 H	7.85, m, 4 H	8.45, m, 2 H	7.67, m, 2 H	8.50, s, 1 H	7.58, s, 1 H							−1150
His	2	14 600	9 400	19 600	28 700	1.53													−1146
AcHis	1	15 800	11 500	23 100	33 500	1.37	8.32, m, 3 H	7.82, m, 4 H	8.45, m, 2 H	7.66, m, 2 H	8.50, s, 1 H	7.44, s, 1 H	4.55, t, 1 H	3.18, d, 2 H	1.96 s, 3 H				
His-His[b]	3	11 900	8 400	19 000	27 800	1.42													
His-Lys[b]	3	13 900	8 700	18 300	27 400	1.60													
Gly-His-Gly	2	13 700	10 400	22 000	30 800	1.32	8.41, m, 3 H	7.84, m, 4 H	8.43, m, 2 H	7.73, m, 2 H	8.52, s, 1 H	7.55, s, 1 H				3.82, m, 4 H	3.46, m, 2 H	*e*	
Free Ligand																			
Im											7.73, s, 1 H	7.09, s, 1 H							
AcHis											7.67, s, 1 H	6.90, s, 1 H	4.40, t, 1 H	3.00, d, 2 H	1.94, s, 3 H				
Gly-His-Gly											7.82, s, 1 H	7.01, s, 1 H				3.75, m, 4 H	3.13, m, 2 H	4.70, t, 1 H	

[a] For 10 μM solutions in water; the wavelengths are in nm. [b] The uncoordinated side chain is assumed to be protonated. [c] For solutions in D_2O at 22 °C. Chemical shifts with respect to the residual water as an internal reference (4.79 ppm vs. DSS as a standard). Nonuniformity of pH in the sample solutions may cause small errors in the chemical shifts. Atoms are numbered as in Scheme I. [d] ^{195}Pt NMR chemical shift for 15–30 mM solutions in 25% D_2O, 75% H_2O, with respect to K_2PtCl_4 as an external standard. [e] Signal of H^{13} is buried under the HDO peak.

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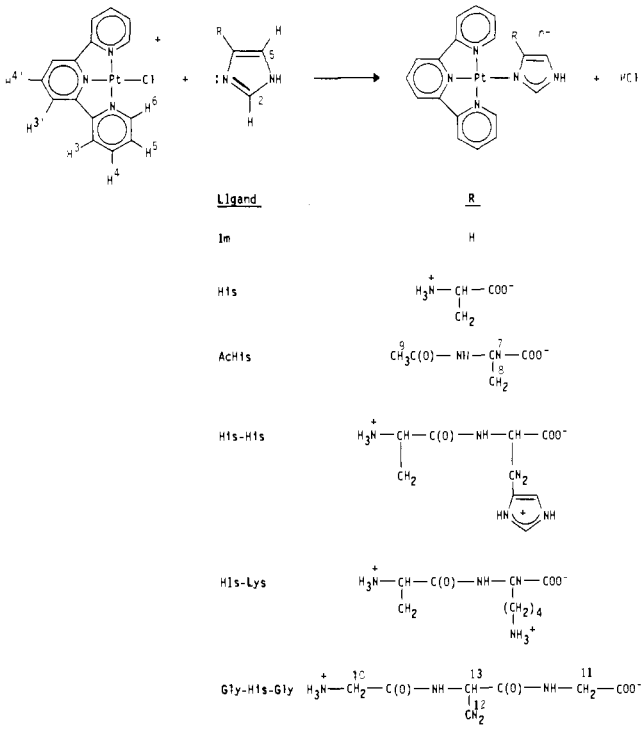
Scheme I

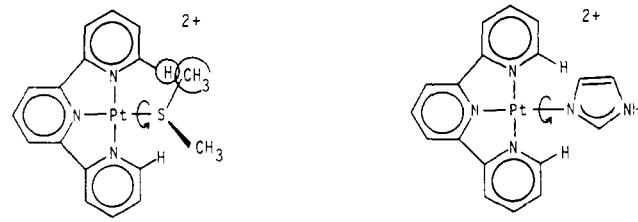


shown in Scheme I. Since all of the $Pt(trpy)L^{2+}$ complexes exhibit virtually identical UV–vis and ^1H NMR spectra (see Table I), all of the ligands must be bonded solely through the imidazole ring. Since the pyrrole-type nitrogen atom is highly basic (pK_a = 14.5 in imidazole),[50] it remains protonated in our experiments. Evidently, the ligating atom is the pyridine-type nitrogen, whose pK_a value is 6.0–6.5.[50] The increase of the k_{obsd} with increasing pH around this value supports the conclusion.

As Table I shows, all of the ^{195}Pt chemical shifts fall in the region characteristic of $Pt^{II}N_4$ complexes.[51] The signals appear about 70 ppm upfield from that of $Pt(trpy)Cl^+$, as expected upon displacement of the Cl^- ligand by a nitrogen donor.[52] The ^1H NMR signals were assigned on the basis of NMR titrations of $Pt(trpy)Cl^+$ with the ligands and of pH titrations of formed complexes. These assignments agree with previous analyses of (terpyridine)platinum complexes[13] and of the free amino acids and peptides.[53–55] Coordination is invariably accompanied by a large movement downfield of the H^6 signal and by slight such movements of the other trpy signals. Similar changes, observed in $Pt(trpy)SR^+$ complexes, were attributed to the magnetic anisotropy of the Pt–S bond,[13] and it is possible that the new Pt–N bond may have a similar effect. The imidazole signals move downfield because of the electron-attracting power of the Pt(II) complex.[56,57] The aliphatic H atoms in the main chain, distant from the donor atom, are not affected significantly.

The electronic absorption spectra, presented in Table I, are particularly interesting. The bands at 242 and 270 nm correspond to transitions in the aromatic trpy ligand. Those at 328 and 342 nm can be attributed to metal-to-ligand charge-transfer transitions

Scheme II



on account of their intensity, dependence on the fourth ligand, and sensitivity to the exogenous species in solution.[32] Although all the complexes contain imidazole as the fourth ligand, their $\epsilon_{342}/\epsilon_{328}$ quotients differ. The main chain, attached to the imidazole ring, evidently affects the chromophore: by direct interaction or by altering the solvation of the complex. The relative band intensities proved diagnostic of the labels differently located on the protein, as will be discussed below.

Unreactivity of Methionine and Its Derivatives. Six thio ethers (methionine and its derivatives) and two disulfides (cystine and oxidized glutathione) failed to displace the Cl^- ligand from $Pt(trpy)Cl^+$ even under forcing conditions. Their unreactivity is surprising in view of the affinity of the soft, nucleophilic thio-ether ligands toward the soft, electrophilic Pt(II) atom,[58–61] especially since the trpy ligand accelerates the displacement of the fourth ligand; for example, $Pt(trpy)Cl^+$ is approximately 10^3–10^4 times more reactive than its aliphatic homolog, $Pt(dien)Cl^+$, toward various small nucleophiles.[62]

Whereas methionine, *S*-methylcysteine, and oxidized glutathione are unreactive, homocysteine, cysteine, and reduced glutathione displace the Cl^- ligand fast.[49] These thiols react rapidly even in the neutral and weakly acidic solutions, in which the SH group (pK_a = 8.3) is protonated. Although the anionic thiolate anion is more nucleophilic than the thio ether or disulfide, the contrast between reactivity and inertness cannot be ascribed solely to the difference in nucleophilicity. We attribute the unreactivity of the biological thio ethers and disulfides to the bulkiness of their respective $RSCH_3$ and RSSR functional groups.[63] Since steric effects on the rate of substitution at Pt(II) are known,[65] we examined molecular models. The pentacoordinate $Pt(trpy)Cl(SMe_2)^+$, representing the putative intermediate or the transition state of the reactions that failed to occur, does not seem to be prohibitively crowded, but the expected product, $Pt(trpy)SMe_2^{2+}$, seems to be. The origin of this steric hinderance was examined in a quantum-chemical study, which is summarized below.

Electronic Structure and Bonding. Molecular orbital calculations of $Pt(trpy)SMe_2^{2+}$ were performed with Pt–S torsion angles of 0, 22.5, 45, 67.5, and 90°. In each case total overlap population between the Pt and S atoms is 0.44 to 0.47 e, corresponding to a normal metal–ligand bond. Regardless of the torsion angle, however, the contacts between a methyl group of the thio ether and an ortho H atom of a pyridine ring are far shorter than the sum of the van der Waals radii; see Scheme II. These repulsions between the filled C–H orbitals, a quantum-mechanical equivalent of steric crowding, outweigh the Pt–S attraction and cause net repulsion between the $Pt(trpy)^{2+}$ and the thio ether. On the contrary, net attraction seems to exist between the $Pt(trpy)^{2+}$ and Im fragments at all Pt–N torsion angles although the planar conformation exhibits some crowding.

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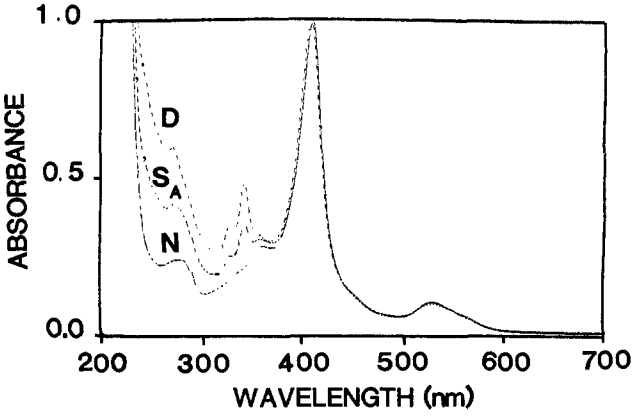

Figure 1. Electronic absorption spectra of the native (N) horse-heart cytochrome *c* and of its derivatives designated S_A and D. The former is singly labeled with a $Pt(trpy)^{2+}$ chromophore at the site marked A (His 33), whereas the latter is doubly labeled at sites marked A (His 33) and B (His 26).

Calculations of $PtCl_3(SMe_2)^-$ and $PtCl_3Im^-$ complexes reveal an absence of steric interactions because the Cl^- ligands are sufficiently small. The difference between trpy and Cl^- will be invoked below to show how ancillary ligands may alter the selectivity of metal complexes toward proteins.

IV. Binding Sites on the Cytochromes

Model Complexes, Absorption Spectra, and Comparison of the Horse and Tuna Proteins. Of all the amino acids that contain heteroatoms in the side chain, only cysteine and histidine proved reactive toward $Pt(trpy)Cl^+$ under the conditions of protein labeling. Since cytochromes *c* from horse and tuna lack free cysteine, the likely binding sites in these proteins are imidazole rings.

The absorption spectra of the native (N), major singly-modified (S_A), and doubly-modified (D) cytochromes *c* from horse are shown in Figure 1. The characteristic absorption bands of the $Pt(trpy)Im^{2+}$ chromophore at 328 and 342 nm are clearly evident. In this region, fortunately, the absorbance of the protein itself is low. Comparison of the difference spectra in Figure 2a with the spectrum of an equimolar solution of $Pt(trpy)(Gly\text{-}His\text{-}Gly)^{2+}$ in Figure 2b confirms that both sites A and B are histidine residues. The spectrum in Figure 2c, showing how markedly the fourth ligand affects the $Pt(trpy)^{2+}$ chromophore, supports the conclusion. The spectra confirm that the S and D derivatives contain one and two labels per protein molecule, respectively. The difference D–N is the sum of the differences S_A–N and S_B–N, i.e., the labels in the doubly-modified derivative are the same two that are found separately in the singly-modified derivatives. The chromophores at histidine residues designated A and B exhibit the same band positions, characteristic of the imidazole ligand, but different intensities: the $\epsilon_{342}/\epsilon_{328}$ quotient is 1.58 in S_A and 1.15 in S_B. This dependence of the band intensities on the ligand structure, already evident among the model $Pt(trpy)L^{2+}$ complexes (Table I), may render the $Pt(trpy)^{2+}$ tag applicable as a probe of its environment in biomolecules.

The horse protein contains histidine in positions 18, 26, and 33. Binding to residue 18, an axial ligand to the heme, can be ruled out. The ferriheme absorption bands at 410 and 530 nm[40,41] and the one at 695 nm, which is dependent on the interactions between the Fe atom and the axial ligands (Met 80 and His 18),[66-68] are unperturbed by labeling. The reduction potential, EPR *g* values, and hyperfine ^1H NMR shifts all retain their "native" values.

Both residues His 33 and His 26 lie on the protein surface but in different environments.[69-72] The former belongs to a hydrophilic region and is exposed to the exterior, whereas the latter is hydrogen-bonded in a hydrophobic pocket[73] and comparatively unreactive.[73-78] Since these two sites are unequally accessible

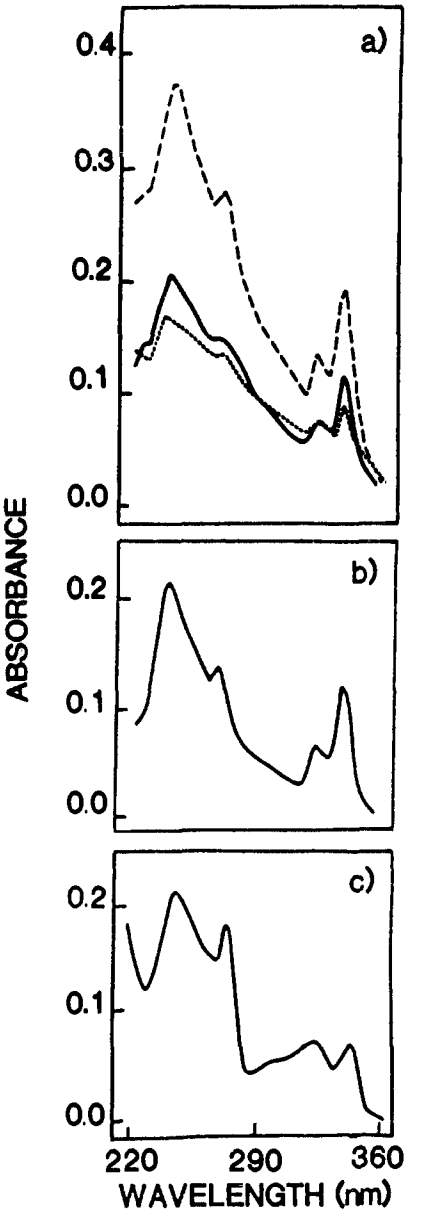

Figure 2. (a) Difference absorption spectra obtained by subtraction of the spectrum of the native (N) horse-heart cytochrome *c* from the spectra of the respective protein derivatives designated S_A, S_B, and D, which are tagged with $Pt(trpy)^{2+}$ chromophore: singly at the sites marked A (in S_A) or B (in S_B) and doubly at both sites (D). The sites A and B are His residues 33 and 26, respectively. (b) Absorption spectrum of $[Pt(trpy)(Gly\text{-}His\text{-}Gly)]Cl_2$, a complex wherein the tripeptide is coordinated to the Pt atom through the imidazole ring in the side chain. (c) Absorption spectrum of [Pt(trpy)Cl]Cl. All the spectra were obtained with 10 μM solutions of the respective proteins and model complexes in 85 mM phosphate buffer at pH 7.0.

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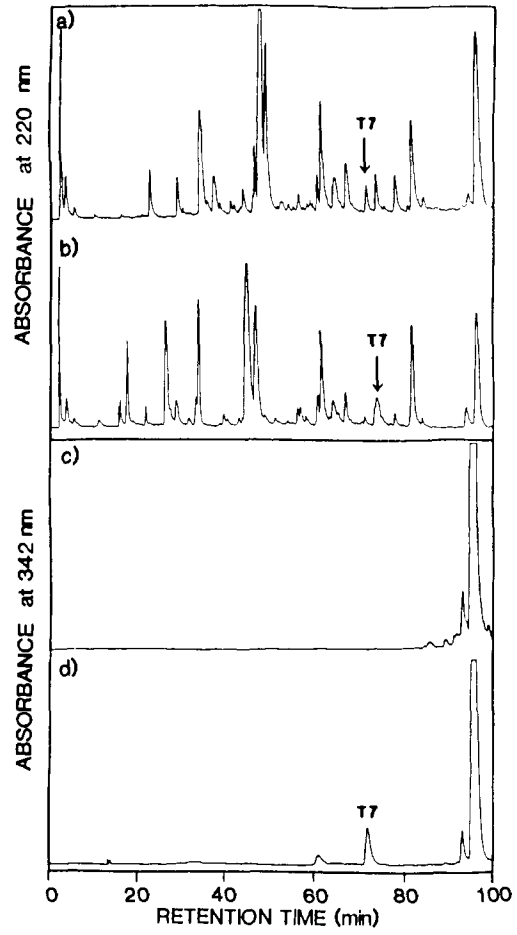

Figure 3. Reversed-phase HPL chromatograms of tryptic digests of the following: (a) and (c), the native horse-heart cytochrome *c*; and (b) and (d), its derivative designated S_A, which is singly labeled with $Pt(trpy)^{2+}$ at the site marked A (His 33). Note the wavelengths at which the absorbances are recorded. The small doublet at 18 min in the chromatograms (b) and (d) is due to the partial dissociation of the label from the major site and subsequent attachment to the minor one in the course of digestion.

to external reagents, the major one (A) can tentatively be identified as His 33 and the minor one (B), as His 26. The difference between the $\epsilon_{342}/\epsilon_{328}$ quotients for the respective $Pt(trpy)^{2+}$ chromophores probably reflects the difference between the site environments.

This assignment is confirmed by the experiments with tuna cytochrome *c*, which contains His 26 but lacks His 33 and has tryptophan, an amino acid unreactive toward $Pt(trpy)Cl^+$, in its place. Since the tuna protein does not yield the major derivative, corresponding to S_A, the site A in the horse protein clearly is His 33. The minor derivative, the only one obtained, closely resembles the S_B derivative of the horse protein by the $\epsilon_{342}/\epsilon_{328}$ quotient and by its elution behavior on the CM 52 column. The minor site B in both cytochromes must then be His 26.

The two sites (in D) are tagged with a greater yield, 7%, than the minor site (in S_B) alone, 2%, because the derivative D may be formed from either of the derivatives S_A or S_B. Since tagging of His 33 (in S_A) perhaps causes a minor conformational change in the protein segment containing His 26 (see section V), subsequent tagging of His 26 to form D may be enhanced by cooperativity.

Elution Behavior. The order of elution from the cation exchanger confirms the assignment of the binding sites. As the number of the cationic $Pt(trpy)^{2+}$ tags increases from none (in N) to one (in S_A and S_B) to two (in D), the protein derivatives become more retained. Although S_A and S_B have the same overall charge, they separate completely on a short column because of their different charge distributions.[31,79] The positive "patches" on the protein surface that are known to interact with the CM 52 resin[31,79,80] are remote from His 33, but one of them lies close to His 26. This is why the derivative S_B, labeled at His 26, is retained more than S_A, labeled at His 33.

Table II. Reduction Potentials and EPR *g* Values of Horse-Heart Cytochrome *c* and of Its Derivatives Labeled with $Pt(trpy)^{2+}$

		labeled at		
	native	His 33	His 26	His 33 and His 26
$E°$, in mV vs. NHE[a]	256 ± 3	255 ± 7	245 ± 6	247 ± 6
EPR[b]				
g_x	1.3	1.3	1.2	1.3
g_y	2.24	2.22	2.27	2.27
g_z	3.00	3.04	3.03	3.00

[a] Differential-pulse voltammetry at 0.1–0.4 mM solutions of the respective proteins, also 10 mM in 4,4-dipyridyl and 100 mM in $NaClO_4$, in 85 mM phosphate buffer at pH 7.0 and 25 °C. [b] Measured with 0.1–1.0 mM solutions of the respective proteins in 85 mM phosphate buffer at pH 7.0 and 6 K.

Peptide Mapping. The undecapeptide T7, which contains His 33,[40] is eluted after 70 min from the "native" digest (see Figure 3a). Retardation to 75 min of the corresponding peptide from the S_A digest (see Figure 3a,b,d) can be attributed to the presence of a Pt label in the derivative. The model undecapeptide Thr-Gly-Pro-Asn-Leu-His-Gly-Leu-Phe-Gly-Arg, which is similar to T7 by composition and hydrophobicity, becomes similarly retarded upon labeling. The difference is small (several minutes) because only one out of eleven residues is modified. Finally, the absorption spectrum of peptide T7 from the derivative S_A shows a $Pt(trpy)His^{2+}$ chromophore. Clearly, His 33 is the major binding site in the horse cytochrome *c*.

The dipeptide T6, which contains His 26, is His-Lys.[40] It is eluted at 2 min from the "native" digest but at 18 min from the S_B digest. In this case the retardation is great, because one out of two residues is modified. A similar difference exists between the authentic dipeptide His-Lys, which elutes at 2.3 min, and its labeled derivative, which exhibits a doublet of peaks at 18.3 and 20.5 min.[81] The general agreement between the retention times confirms His 26 as the minor binding site in the cytochrome *c*. The two binding sites are shown in Figure 4.

Selectivity in Binding. Compound $Pt(trpy)Cl^+$ reacts with proteins under mild conditions—in equimolar ratio at room temperature. Its specificity (at pH 5) toward histidine was confirmed by thorough studies of model reactions with amino acids, their derivatives, and peptides. This specificity remains even when a thiol group is present. Although cytochrome *c* from baker's yeast contains reactive Cys 102, this protein and the homologous one from *Candida krusei* are labeled exclusively at histidine residues.[82]

Substitution reactivity of $Pt(trpy)Cl^+$ is opposite from that of another platinum(II)–chloro complex, the common $PtCl_4^{2-}$. Whereas the latter is highly reactive toward methionine and barely so toward histidine,[8,9,11] the former proved totally unreactive toward methionine and very reactive toward histidine. The reversal can be attributed to the properties of the terpyridine ligand. By its steric demands, it prevents binding to the thio ether, which is otherwise highly reactive toward Pt(II) complexes. By its electronic properties, however, it facilitates the displacement of Cl^- even by imidazole, which is otherwise comparatively unreactive.

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(81) This doubling is characteristic of the Pt(trpy)L complexes with small ligands. We observed it with imidazole, histidine, and dipeptide His-Lys but not with longer peptides. The component at 20.5 min may be delayed by some nonspecific interaction between the Pt(trpy) unit and the reversed-phase chromatographic column.
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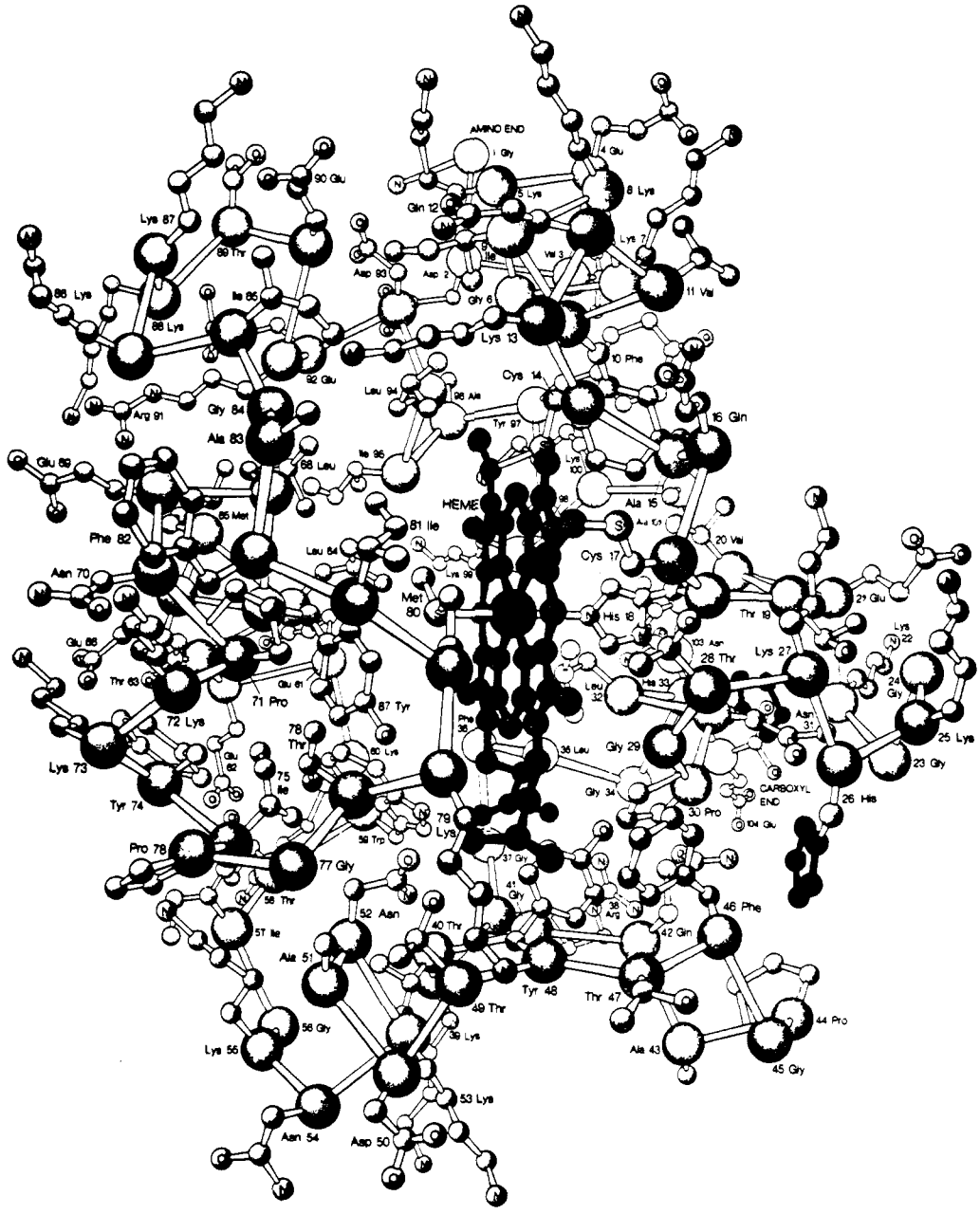

Figure 4. Amino-acid residues in cytochrome *c* whose side chains react with $Pt(trpy)Cl^+$ at pH 5.0. In horse-heart protein, shown here, both positions 26 and 33 are occupied by histidine residues. Their respective imidazole side chains are shown in black: that of His 26 to the right and that of His 33 in the back right. In this picture, based on ref 69, the heme plane is viewed obliquely from the left side, that of the Met 80 axial ligand to iron. Copyright 1972: Dickerson, R. E.; Geis, I. *Sci. Am.* **1972**, *226*, 62.

The specificity of transition-metal reagents toward biomolecules evidently can be controlled by the judicious choice of ancillary ligands.

V. Structural and Redox Properties of the Modified Proteins

Various physical methods were applied to determine whether labeling with the novel reagent alters the properties of cytochrome *c*. The absorption spectra indicate that the electronic structure of the heme is not noticeably perturbed. The electrochemical data in Table II show no significant change of the reduction potentials either. The redox processes are quasi-reversible, with CV peak separations of 75 mV. The peak current in each case increases linearly with the square root of the scan rate, an indication of a diffusion-controlled one-electron process.[42] The EPR *g* values, presented in Table II, agree with those for the native cytochrome *c*.[83,84]

The proton NMR spectrum of the paramagnetic ferriheme depends markedly on the interactions between the iron atom and its axial ligands and between the heme periphery and the neighboring amino-acid residues.[85-94] Particularly sensitive to these

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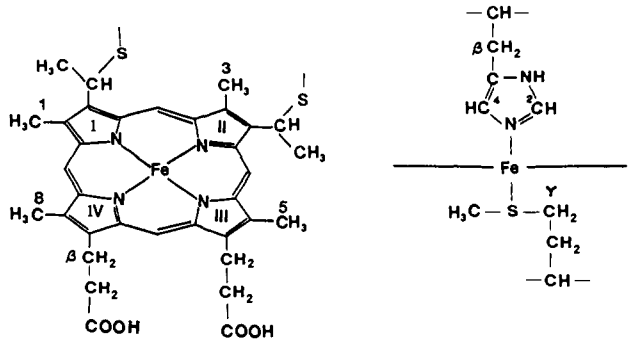
Table III. The Hyperfine-Shifted ^1H NMR Signals of Horse-Heart Cytochrome *c* and of Its Derivative Labeled with $Pt(trpy)^{2+}$ at His 33[a]



chmcl shift,[b] ppm			pertrbtn,[c]	
native	labeled	assignment	ppm	ref
35.73	35.73	CH_3 no. 8	0	88, 91–94
32.69	33.03	CH_3 no. 3	+0.34	88, 91–94
24.2	24.2	S–C*H*–CH_3 in II; or CH no. 2 and CH no. 4 of His 18	0	91–92
19.22	18.71	βCH_2 of propionate in IV	−0.51	91–94
14.64	14.46	βCH_2 of His 18	−0.18	92
12.81	12.81	unassigned	0	92
11.47	11.71	βCH_2 of propionate in IV	+0.24	92
9.89	9.89	CH_3 no. 5	0	91–94
6.81	6.81	CH_3 no. 1	0	91–94
−2.53	−2.53	S–CH–C*H*$_3$ in II	0	91–94
−2.82	−2.82	S–CH–C*H*$_3$ in I	0	91–94
−4.48	−4.48	meso CH of heme	0	94
−6.39	−6.39	meso CH of heme	0	94
−24.70	−24.70	CH_3 of Met 80	0	88, 93
−28.1	−28.1	γCH_2 of Met 80	0	94

[a] Measured with 2–4 mM solutions of the respective proteins in 85 mM phosphate buffer at pH 7.0 and 21 °C. [b] With respect to DSS as an internal standard. For accuracy in control experiments, the labeled protein, S_A, was spiked with the native protein. [c] Defined as $\delta_{labeled} - \delta_{native}$.

interactions are the hyperfine ^1H shifts.[41] As Table III shows, labeling leaves them virtually unperturbed. Only the shifts of the CH_3 group at the pyrrole ring II and of the β-CH_2 group in the propionate chain at the ring IV are altered by more than ±0.25 ppm.[89] The ring II is connected with the protein through Cys 17; attachment of a $Pt(trpy)^{2+}$ label to His 33 perhaps causes a slight movement in the polypeptide backbone, which may be transmitted to the pyrrole ring II. This hypothesis of the correlated motions of the backbone and the heme is borne out by comparisons between the structures of ferro- and ferricytochrome *c*.[95,96] Crystallographic studies revealed that the change in the oxidation state is accompanied by the movement not only of the heme but also of the polypeptide chain between residues 17 (the Cys link) and 33 (the labeled His in the horse protein), exactly the segment implicated in our hypothesis. The slight perturbation of the β-CH_2 group may be due to a direct electrostatic attraction between the propionate and the $Pt(trpy)^{2+}$ label. An indirect interaction, too, is conceivable: labeling of His 33 perhaps causes a slight movement of the proximate Tyr 48, which is hydrogen-bonded to the propionate chain at the ring IV.[95]

VI. Advantages of the New Labeling Reagent

Complex $Pt(trpy)Cl^+$ is well suited for tagging biological macromolecules on account of its reactivity, selectivity, stability, and spectroscopic properties. Owing to an interplay between the steric and electronic effects of the ancillary terpyridyl ligand, the selectivity of $Pt(trpy)Cl^+$ is entirely opposite from that of the common reagent, $PtCl_4^{2-}$. Stability of the $Pt(trpy)^{2+}$ tags permits storage, dialysis, and even cation-exchange chromatography of the modified proteins. The tags can be removed, however, and the native protein restored easily, by treatment with highly nucleophilic ligands.

Perhaps the greatest advantage of the new reagent lies in the strong absorption bands of the $Pt(trpy)L^{n+}$ chromophore, characterized by the extinction coefficients of 13 000–30 000 M^{-1} cm^{-1}, which permit easy detection and quantitation. The charge-transfer bands in the region of 320–350 nm, unobscured by the protein absorption, are sensitive not only to the nature of the binding site but also to its environment. Findings with cytochrome *c* indicate that even multiple labeling does not perturb protein properties.

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