interactions are particularly effective in destroying the typical one-dimensional line shape, corresponding to the Fourier transform of $\Phi = \exp(-\gamma t)^{3/2}$, restoring the Lorentzian line shape of exchange-narrowed systems.[38]

On a quantitative basis this is monitored by a characteristic time t_0, at which interchain flips become important. The order of magnitude estimating for one-dimensional spin systems is

$$t_0^{-1} \cong \frac{J'}{h}(J'/J)^{1/3}$$

Numerical calculations[38] showed that notable deviations from Lorentzian line shape can be observed when $J'/J \leq 10^{-2}$. This may be considered to be a very high upper limit for the J'/J ratio, since we do not observe transitions to three-dimensional order above 10 K, suggesting that $J'/J \leq 10^{-3}$.

If we consider that every chain is surrounded by six other chains with the shortest contact of 9.43 Å, the small value of J' is not surprising. This is a desirable property if one is interested in monodimensional behavior, but it is much less appealing if the purpose is designing compounds with bulk magnetic properties. Clearly, although strong intrachain interaction can be obtained by using metal ions and radicals, it is necessary to introduce groups that can enhance interchain interactions in order to have relatively high temperature magnetic materials. Nevertheless it is certainly encouraging that the transition to three-dimensional order seems not to be of the antiferromagnetic type.

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Registry No. $Mn(hfac)_2NITPh$, 113533-14-5; $Mn(hfac)_2NIT$-*i*-Pr, 113547-96-9; $Mn(hfac)_2NITEt$, 113533-16-7; $Mn(hfac)_2NITMe$, 113533-18-9; $Mn(hfac)_2$, 19648-86-3.

Supplementary Material Available: Tables of derived hydrogen positions (Table SII), anisotropic thermal parameters (Table SIII), complete bond distances (Table SIV), and complete bond angles (Table SV) for $Mn(hfac)_2NIT$-*i*-Pr and plots of experimental χT values vs T for II (Figure S1), III (Figure S2), and IV (Figure S3) (10 pages); a table of observed and calculated structure factors (6 pages). Ordering information is given on any current masthead page.

Contribution from the Department of Chemistry and Ames Laboratory, Iowa State University, Ames, Iowa 50011

Noninvasive Tagging of Proteins with an Inorganic Chromophore. Selectivity of Chloro(terpyridine)platinum(II) toward Amino Acids, Peptides, and Cytochromes *c*

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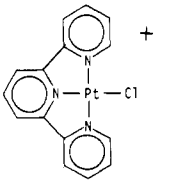
The complex $[Pt(trpy)Cl]^+$ exhibits unexpected selectivity toward amino acid side chains in cytochromes *c* from *Candida krusei* and bakers' yeast. Although kinetic studies with amino acids and peptides as entering ligands prove this complex to be completely selective toward thiol over imidazole, His-33 and His-39 residues (in both proteins) are labeled with greater yields than the Cys-102 residue (in the bakers' yeast protein). The binding sites are determined by peptide mapping and other methods. The $Pt(trpy)^{2+}$ tags are stable, and the protein derivatives are separated by cation-exchange chromatography. The $[Pt(trpy)His]^{2+}$ and $[Pt(trpy)Cys]^+$ chromophores are easily detected and quantitated owing to their characteristic and strong UV–vis bands. Spectroscopic and electrochemical measurements show that labeling with the new reagent does not alter the structural and redox properties of the cytochromes *c*. The unexpected outcome of the protein labeling indicates that, contrary to the common assumption, Cys-102 is not exposed at the protein surface. Modification of this residue with various organic reagents and dimerization of the protein must be accompanied by a perturbation of the conformation, which makes Cys-102 accessible to the reagent or to another molecule of the protein. These predictions from the labeling study are confirmed subsequently by the crystallographic study of the *iso*-1 cytochrome *c* from bakers' yeast. The inorganic complex $[Pt(trpy)Cl]^+$ differs from the other reagents for protein modification by its noninvasiveness, a property that may well render it useful as a probe of the protein surface.

I. Introduction

Covalent modification of amino acid side chains has proved very useful in structural, spectroscopic, and mechanistic studies of proteins.[1-3] Various spin labels, chromophores, fluorescent probes, and radioactive labels developed to date are mostly organic compounds. Except as heavy-atom scatterers in crystallographic studies,[4,5] metal complexes have not been used widely for covalent modification of proteins although their properties are well suited for this purpose.[6,7] Transition-metal complexes can serve as absorption chromophores, as paramagnetic EPR labels, as NMR probes and relaxation agents, and as electron-transfer reagents. Selectivity in binding can be controlled by the oxidation state, hardness or softness, coordination number, ancillary ligands, and charge.

Previous research in this laboratory showed that chloro(2,2′:6′,2″-terpyridine)platinum(II), $[Pt(trpy)Cl]^+$, reacts specifically with histidine (His) residues in cytochromes *c* from horse and tuna at pH 5.0.[8] The $Pt(trpy)His^{2+}$ complex, formed by



$[Pt(trpy)Cl]^+$

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Table I. Absorption Spectra of $[Pt(trpy)L]^{n+}$ Complexes, Models for Tags on Proteins

L	n^a	absorptivity, ϵ, of 10 μM aqueous solution, M^{-1} cm^{-1}						
		342 nm	328 nm	311 nm	278 nm	270 nm	242 nm	$\epsilon_{342}/\epsilon_{328}$
$^-SCH_2CH_2OH$	1	12 800	11 200	11 200	19 500		27 800	1.14
Cys	1	14 200	12 400	12 100	21 500		30 300	1.14
γ-Glu-*Cys*-Gly[b]	0	14 100	12 600	12 500	21 800		30 900	1.12
Im[c]	2	16 000	10 400			21 200	31 800	1.54
His[c]	2	14 600	9 400			19 600	28 700	1.53
Gly-*His*-Gly[c]	2	13 700	10 400			22 000	30 800	1.32

[a] When the thiol, imidazole, and carboxyl groups are deprotonated and the amino groups are protonated. [b] Glutathione. [c] Reference 8.

displacement of the Cl^- ligand by the pyridine-like N atom of imidazole, is easily detected and quantitated on account of its characteristic and strong UV–vis absorption spectrum. Because of an interplay between the steric and electronic effects of the terpyridyl ligand, the new reagent is unreactive toward methionine and cystine, which are otherwise very strong ligands, but very reactive toward histidine, which is otherwise a relatively weak ligand. Judiciously chosen ancillary ligands, in this case terpyridyl, evidently can determine selectivity of transition-metal complexes toward biological macromolecules.

Our studies with amino acids and peptides showed $[Pt(trpy)Cl]^+$ to be more reactive toward cysteine (Cys) than toward histidine, as expected of a soft Pt(II) atom.[9-11] Since neither of the proteins examined before contains a free cysteine residue, the observed selectivity toward histidine seemed fortuitous. In this study, the selectivity of $[Pt(trpy)Cl]^+$ is tested stringently in reactions with two additional cytochromes *c*: the *iso*-1 form of the protein from bakers' yeast, which contains the highly reactive residue Cys-102,[12-18] and the protein from *Candida krusei*, which lacks free Cys residues. Both of these homologous proteins also contain, among other residues, the same set of histidines as potential binding sites.

II. Amino Acid and Peptide Complexes

Absorption Spectra. The model complexes $[Pt(trpy)L]^{n+}$ are shown in Scheme I, and their UV–vis spectroscopic properties are presented in Table I. The bands in the region 300–350 nm can be attributed to MLCT transitions because of their high intensity and their dependence on ligands L and on other species in solution.[11,19] The bands below 300 nm are due to transitions in the aromatic terpyridyl ligand. The relative intensities of the bands, especially of those at 342 and 328 nm, are nearly identical among the complexes with similar ligands L, but markedly different between the complexes with different ligands, such as imidazole and thiolate. Job's plots[11] show that each of the tripeptides γ-Glu-Cys-Gly (glutathione) and Gly-His-Gly has only one group that is reactive toward $[Pt(trpy)Cl]^+$—thiol and imidazole, respectively. The terdentate trpy ligand obviously prevents multidentate binding of other ligands.

Kinetics of Displacement Reactions. The reactions shown in Scheme I were followed under pseudo-first-order conditions, with a 100-fold excess of entering ligands. The rate constants in Table II show that thiol ligands react approximately 300 times faster than imidazole ligands under identical conditions. A natural consequence of this difference in rate is the complete selectivity of $[Pt(trpy)Cl]^+$ for γ-Glu-Cys-Gly over Gly-His-Gly. When the two tripeptides were allowed to compete for the Pt complex, both the rate constant k_{obsd} and the absorption spectrum characteristic of the glutathione complex were obtained. The observed selectivity is consistent with the large difference between thiolate and imidazole ligands in their nucleophilicity toward Pt(II) complexes; the respective n_{Pt} parameters are 7.17 and 3.44.[20] The selectivity remains although, at pH 7.0, the concentration of deprotonated imidazole (pK_a = 6.0 in His) is 18 times larger than that of deprotonated thiolate (pK_a = 8.3 in Cys).[21]

Scheme I

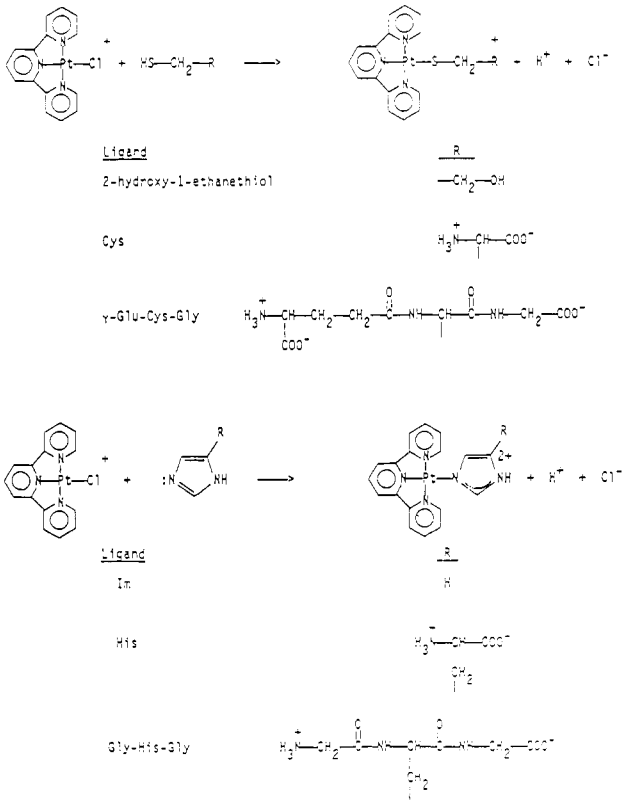

Table II. Pseudo-First-Order Rate Constants for Reactions Shown in Scheme I[a]

ligand	k_{obsd}, s^{-1}
$HSCH_2CH_2OH$	$(2.5 \pm 0.3) \times 10^{-2}$
Cys	$(1.3 \pm 0.3) \times 10^{-2}$
γ-Glu-*Cys*-Gly	$(5.5 \pm 0.3) \times 10^{-2}$
γ-Glu-*Cys*-Gly and Gly-*His*-Gly[b]	$(1.2 \pm 0.4) \times 10^{-2}$
Im	$(2.5 \pm 0.1) \times 10^{-4}$
His	$(0.85 \pm 0.1) \times 10^{-4}$
Gly-*His*-Gly	$(3.1 \pm 0.1) \times 10^{-4}$
horse heart cyt *c*	$(3.4 \pm 0.4) \times 10^{-4}$
bakers' yeast cyt *c*	$(3.5 \pm 0.4) \times 10^{-4}$

[a] At 25 °C, with 20 μM [Pt(trpy)Cl]Cl and 2 mM entering ligand. [b] Equimolar amounts of competing ligands.

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Stability of [Pt(trpy)L]$^{n+}$ Complexes. Success of peptide-mapping experiments depends on the stability of the Pt(trpy)$^{2+}$ tags at the sites of their initial attachment to the proteins. Migration of the tags during protein digestion or during chromatography of the resulting peptides could cause an erroneous identification of the binding sites. The [Pt(trpy)His]$^{2+}$ complex is stable even at pH 3.0, but since the thiolate is far more nucleophilic than imidazole, the displacement reaction in eq 1 is a

$$[Pt(trpy)His]^{2+} + CysH \rightarrow [Pt(trpy)Cys]^{+} + HisH^{+} \quad (1)$$

very facile and rapid one. (The symbol H denotes protonation of the side chain.) The reaction in eq 1 is fast even at pH 2.0, at which the concentration of the thiolate form is negligibly low. Standard peptide-mapping procedures proved inadequate for the bakers' yeast cytochrome *c* because the tag did migrate from the His-containing peptides to the Cys-containing one. This migration was prevented by a method based on the following reactions.

Even in the presence of a large excess of $HOHgC_6H_4SO_3^-$, the platinum complex displaces the organomercurial reagent from the cysteinato compound of the latter, according to eq 2. The reaction

$$[Pt(trpy)Cl]^{+} + CysHgC_6H_4SO_3^- \rightarrow [Pt(trpy)Cys]^{+} + ClHgC_6H_4SO_3^- \quad (2)$$

is rapid and the UV–vis spectrum showed [Pt(trpy)Cys]$^+$ to be the only platinum complex present. The affinity of [Pt(trpy)Cl]$^+$ toward cysteine side chain evidently is extremely high, higher even than the well-known affinity of the organomercurial reagents, which are commonly used for cysteine modifications. Therefore, the reaction in eq 3 does not happen.

$$[Pt(trpy)Cys]^{+} + HOHgC_6H_4SO_3^- \not\rightarrow [Pt(trpy)OH]^{+} + CysHgC_6H_4SO_3^- \quad (3)$$

A similar reaction, shown in eq 4, involving [Pt(trpy)His]$^{2+}$ does not happen. Fortunately, this organomercurial reagent cannot displace the platinum labels from the His residues, eq 5.

$$[Pt(trpy)His]^{2+} + CysHgC_6H_4SO_3^- \not\rightarrow [Pt(trpy)Cys]^{+} + HisHgC_6H_4SO_3 \quad (4)$$

$$[Pt(trpy)His]^{2+} + HOHgC_6H_4SO_3^- \not\rightarrow [Pt(trpy)OH]^{+} + HisHgC_6H_4SO_3 \quad (5)$$

These experiments show that the Pt(trpy)$^{2+}$ tag can migrate from His to free Cys (eq 1) but not to mercurated Cys (eq 4). Treatment of the Pt(trpy)$^{2+}$-modified protein with $HOHgC_6H_4SO_3^-$ prior to tryptic digestion therefore ensures the fidelity of peptide mapping, as will be discussed in section IV.

III. Cytochrome *c* Complexes

Synthesis. Labeling of the proteins occurs easily under mild conditions—incubation with an equimolar amount of [Pt(trpy)Cl]$^+$ for 1 day. After dialysis of the unbound Pt reagent, the protein is chromatographed on CM 52 with phosphate buffer at pH 7.0 as an eluent. The greater the number of the cationic Pt(trpy)$^{2+}$ labels covalently attached to the protein, the slower the elution of the derivative from the cation exchanger. The chromatographic bands of the two proteins are designated with the initials of the corresponding organisms: C for *C. krusei* and Y for (bakers') yeast. The *C. krusei* cytochrome *c* yielded, in addition to the native protein (band C1), three fractions: two singly labeled (bands C2 and C3) derivatives and one doubly labeled (band C4) derivative. The yeast protein yielded, in addition to the native protein (band Y1), six fractions: three singly labeled (bands Y2, Y3, and Y4) and three doubly labeled (bands Y5, Y6, and Y7) derivatives. The native and singly labeled fractions are eluted with the 100 mM buffer, and the doubly labeled ones required higher ionic strength, achieved by the addition of NaCl. Despite many attempts, separation of fractions Y3 and Y4 and complete separation of fractions C2 and C3 was not achieved. The yields are listed in Table III.

The representative absorption spectra of the native and labeled cytochromes *c* from *C. krusei* are shown in Figure 1. The characteristic strong absorption bands of the [Pt(trpy)His]$^{2+}$ chromophore at 328 and 342 nm are prominent in the region in which the protein absorbance is low. The presence of one or two labels per protein molecule is clearly evident. Similar spectra are obtained with the yeast protein. The number of singly and doubly labeled derivatives indicates the presence of two binding sites in the *Candida* protein and three such sites in the yeast protein.

Table III. Products of Reactions between Cytochromes *c* and [Pt(trpy)Cl]$^+$, Separated by Cation-Exchange Chromatography

cytochrome *c*	fraction	binding sites	no. of Pt(trpy)$^{2+}$ tags	relative yield, ±3%
horse heart	H4	26 and 33	2	10
	H3	26	1	5
	H2	33	1	50
	H1		0	35
C. krusei	C4	33 and 39	2	10
	C3	39	1	30
	C2	33	1	30
	C1		0	30
bakers' yeast	Y7	His-33 and His-39	2	5
	Y6	Cys-102 and His-33	2	5
	Y5	Cys-102 and His-39	2	5
	Y4	His-39	1	20
	Y3	His-33	1	15
	Y2	Cys-102	1	10
	Y1		0	40

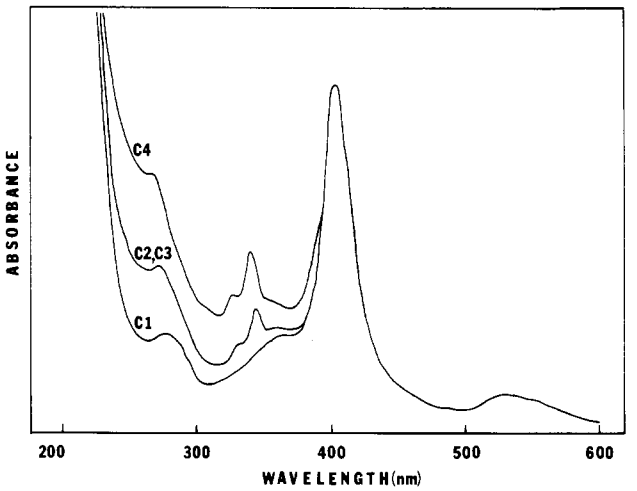

Figure 1. Absorption spectra: native cytochrome *c* from *C. krusei* (C1); mixture of its derivatives singly labeled with Pt(trpy)$^{2+}$ at His-33 (C2) and at His-39 (C3); derivative doubly labeled at His-33 and His-39 (C4).

Structural and Redox Properties. The modified and native proteins were compared with one another by various physical methods in order to determine whether labeling with the novel inorganic reagent alters the properties of cytochrome *c*. Since the singly labeled derivatives C2 and C3 are only partially resolved, and Y3 and Y4 are unresolved, the measurements involving them are done with the mixtures. The findings are summarized in Table IV. The absorption spectra indicate that the electronic structure of the heme is not noticeably perturbed. The reduction potentials of the native and modified proteins from the same organism, determined by cyclic and differential pulse voltammetry, are nearly identical within the margin of experimental error.[22,23] The similarities among the EPR *g* values rule out significant electronic or structural perturbations of the heme.[24] The evidence against perturbation is reliable even when obtained with a mixture of tagged proteins.

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Table IV. Redox and Spectroscopic Properties of the Native Cytochromes *c* and of Their $Pt(trpy)^{2+}$-Tagged Derivatives[a]

	C. krusei			bakers' yeast			
	C1	C2 and C3[b]	C4	Y1	Y2	Y3 and Y4[b]	Y7
redn. potential, ±5 mV vs NHE[c]	273	275	272	282	287	280	277
EPR[d]							
g_x	1.20	1.20	1.20	1.20		1.30	1.20
g_y	2.23	2.23	2.09	2.16		2.19	2.19
g_z	3.07	3.06	3.18	3.12		3.14	3.12
^1H NMR[e]							
CH_3 at ring IV	33.70	33.73 (0.03)		34.75	34.92 (0.15)	34.25 (0.50)	
CH_3 at ring II	31.02	31.35 (0.33)		30.85	31.12 (0.27)	30.57 (0.18)	
β-CH of propionate at ring IV	16.94	16.55 (0.39)		15.17	15.29 (0.12)	15.01 (0.16)	
β-CH of propionate at ring III	14.19	14.25 (0.06)		14.37	*f*	*f*	
CH_3 at ring III	10.56	10.54 (0.02)		11.65	11.19 (0.46)	11.35 (0.30)	
CH_3 in Met-80	−23.69	−23.29 (0.40)		−22.67	−23.12 (0.45)	−22.39 (0.28)	
S-CH-CH_3 at ring I	−3.19	−3.14 (0.05)		−3.14	−3.06 (0.08)	−3.13 (0.01)	
S-CH-CH_3 at ring II	−2.39	−2.37 (0.02)		−2.26	−2.31 (0.05)	−2.16 (0.10)	

[a] For the designations of the protein derivatives and for the tagged amino acids, see Table III. [b] Mixture of two singly labeled derivatives. [c] Solution composition: 0.1–0.4 mM in the protein, 0.1 M in $NaClO_4$, 0.01 M in 4,4'-bipyridyl, and 0.085 M in phosphate buffer at pH 7.0 and 25 °C. [d] Measured with 0.1–1.0 mM solutions of the proteins in 85 mM phosphate buffer at pH 7.0 and 8 K. [e] Measured with 1–3 mM solutions of the proteins in D_2O, with chemical shifts in ppm downfield from DSS; deviations from the native values are given in parentheses. [f] Not detected.

The ^1H NMR resonances of the paramagnetic ferriheme, especially those shifted by hyperfine effects, depend markedly on the interactions between the iron atom and its axial ligands and between the heme periphery and the neighboring amino acid residues.[25-34] The spectra are assigned according to previous studies.[31,35-38] Labeling evidently leaves the protein structure, at least the features manifest in the four properties measured, virtually unperturbed. Our previous study of cytochromes *c* from horse and tuna[8] and this study both indicate that $[Pt(trpy)Cl]^+$ is a noninvasive reagent for protein modification.

IV. Binding Sites on the Cytochromes *c*

Our qualitative study of reactivity of $[Pt(trpy)Cl]^+$ toward all the amino acids that contain heteroatoms in their side chain showed that only Cys and His are reactive under the conditions of protein labeling.[8] The kinetic experiments described in section II confirm these conclusions quantitatively. Horse and tuna cytochromes *c*, neither of which contains free Cys residues, are modified exclusively at His residues at pH 5.0.[8] The yields of the singly labeled derivatives were proportional to the accessibility of the respective binding sites. In the horse protein the major site is His-33, a residue fully exposed on the protein surface; the minor site is His-26, which is only slightly exposed. The tuna protein lacks His-33 and has tryptophan, an amino acid inert toward $[Pt(trpy)Cl]^+$, in its place; consequently, the binding occurs solely at His-26. The binding sites in the horse and tuna proteins were determined by UV–vis spectrophotometry and peptide mapping. The latter method was augmented with control experiments involving amino acid and peptide complexes as models for the tryptic fragments of the modified proteins.

Potential Sites. Since the heme environment is not perturbed by protein modification (see section III), binding to His-18, an axial ligand to the Fe atom, can be ruled out. The potential binding sites are the following: His-26, His-33, and His-39 in both proteins; and Cys-102 only in the *iso*-1 protein from yeast because the protein *C. krusei* has serine, an amino acid inert toward $[Pt(trpy)Cl]^+$, in position 102.

All the cytochromes *c* under consideration have similar amino acid sequences and are folded in the same, characteristic manner.[39-41] Since the crystal structures of the proteins from horse,[42] tuna,[43-46] and bakers' yeast[47,48] are known and since the protein from *C. krusei* is homologous with them, especially with that from yeast, characteristics of the potential binding sites in all four proteins are known. Polypeptide segments 19–26 and 27–33 retain their conformations in all of the cytochromes *c* of interest.[49] Both His-26 and His-33 lie on the protein surface, but in different environments.[43] The former is hydrogen bonded in a hydrophobic pocket and largely shielded from the exterior, whereas the latter belongs to a hydrophilic region and is exposed to the exterior.[43,50] Residue 39 (Lys in the proteins from horse and tuna, and His in that from yeast) is exposed on the surface.[43,48] These considerations of structure show clearly that His-33 and His-39 are likely and that His-26 is unlikely to react with $[Pt(trpy)Cl]^+$.

Since cytochromes *c* adopt very similar structures in the crystalline state and in solution,[29] exposure of residues can be inferred from labeling studies (among which is our own, mentioned above).[8] Indeed, His-33 and His-39 in the bakers' yeast cytochrome *c* are modified with diazonia-1*H*-tetrazole, whereas His-26 is not.[51] Since Cys 102 in the yeast protein is very reactive toward various compounds[12-18] and since the protein dimerizes easily,[13,14,17] the free thiol is generally considered to be accessible from solution.[16]

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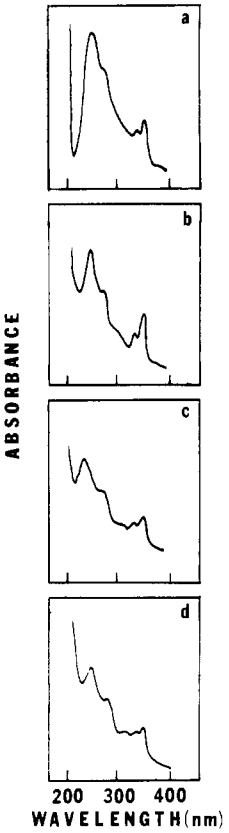

Figure 2. (a) Difference between the absorption spectra of the singly labeled (Y3 and Y4) and native (Y1) bakers' yeast cytochrome *c*. The former is a mixture of derivatives tagged with $Pt(trpy)^{2+}$ at His-33 (Y3) or at His-39 (Y4). (b) Absorption spectrum of [Pt(trpy)(Gly-*His*-Gly)]Cl_2, a complex in which the tripeptide is coordinated through the imidazole ring. (c) Difference between the absorption spectra of the singly labeled (Y2) and native (Y1) bakers' yeast cytochrome *c*. The former contains a $Pt(trpy)^{2+}$ tag at Cys-102. (d) Absorption spectrum of [Pt(trpy)(γ-Glu-*Cys*-Gly)], a complex in which glutathione is coordinated through the thiolate group.

Histidine Residues. Subtraction of the UV–vis spectrum of the native protein from the spectra of its singly tagged derivatives in all cases except in the case of Y2 yields the difference spectrum characteristic of the model complexes with imidazole ligands. One such comparison is shown in Figure 2a,b. Clearly, the binding sites in all the derivatives except Y2 are His residues; consideration of their exposure on the protein surface (see above) points at His-33 and His-39, both of which are accessible from solution.

In our previous study of cytochromes *c* from horse and tuna, the relative intensities of the absorption bands of $[Pt(trpy)His]^{2+}$ proved sensitive to the environment of the His residue to which the chromophore is attached. The tag in the hydrophobic region (at His-26) had a $\epsilon_{342}/\epsilon_{328}$ quotient of 1.15, whereas the one in the hydrophilic region (at His-33) had a quotient of 1.58.[8] In this study, the chromophores attached to both His residues in both proteins had the high $\epsilon_{342}/\epsilon_{328}$ quotient, 1.45 in the *C. krusei* derivatives and 1.50 in the yeast derivatives. Both of the His residues involved seem to be in hydrophilic environments, i.e., exposed to the solution. Indeed, both His-33 and His-39 are located in such environments. Residue His-26, a very minor site in the horse and tuna proteins, is not tagged in the *C. krusei* and yeast proteins because the latter two proteins have a greater number of potential binding sites than the former two. This minor site (His-26) could be labeled slightly in competition with one other site (His-33), but it cannot compete with two other sites (His-33 and His-39).

The minor perturbations of the ^1H NMR resonances of the methyl groups in the heme rings III and IV are evident in the labeled yeast protein but not in the labeled horse[8] and *C. krusei* proteins; see Table IV. The binding sites His-33 and His-39 (especially the former) are near these rings in all the homologous proteins, but the tertiary structure of the first one is less stable[52] than those structures of the other two proteins. Hence the slight perturbations only in the spectrum of the yeast protein.

Cysteine Residue. The singly labeled derivative Y2 of yeast cytochrome *c* yielded a UV–vis difference spectrum characteristic of $[Pt(trpy)Cys]^+$; compare parts c and d of Figure 2. The $\epsilon_{342}/\epsilon_{328}$ quotient is 1.14 for this model complex and 1.28 for the difference spectrum Y2 − Y1. The yield of the tagging at Cys-102 is surprisingly low in view of the high reactivity of this residue toward organic reagents[12-18] and in view of the complete selectivity of $[Pt(trpy)Cl]^+$ for Cys over His in the model reactions.

Peptide Mapping. Tryptic digests of the various labeled proteins—those from the yeast cytochrome *c* were pretreated with $HOHgC_6H_4SO_3^-$—were analyzed by HPLC, and tagged peptides were detected easily owing to the high absorptivity of the $[Pt(trpy)L]^{n+}$ chromophores at 342 nm. Pretreatment with the organomercurial is necessary in order to suppress (as in eq 4) the migration of the $Pt(trpy)^{2+}$ tag from the His (i.e., imidazole) ligand to the Cys (i.e., thiolate) ligand (eq 1). The identity of the tagged amino acid (His or Cys) was clear from the UV–vis spectra of the peptides. The labeled peptide from Y2 was identified on the basis of its UV–vis spectrum, and such peptides from Y3 and Y4 were identified by amino acid analysis. The remaining peptides were identified on the basis of their retention times in comparison with those of the definitely recognized peptides. The assignments were unambiguous because no tryptic peptide contains more than one amino acid reactive toward $[Pt(trpy)Cl]^+$. The protein derivatives, $Pt(trpy)^{2+}$-labeled peptides (designated as usual), and their retention times (in min in parentheses) are as follows: Y2, T20 (52); mixed Y3 and Y4, T8 (35) and T9 (48); mixed C2 and C3, T6 (36) and T7 (44); doubly labeled C4, T6 (36) and T7 (42). Peptides T8, T9, and T20 of yeast cytochrome *c* contain His-33, His-39, and Cys-102, respectively. Peptides T6 and T7 of *C. krusei* cytochrome *c* contain His-33 and His-39, respectively. The reversed-phase HPLC experiments with protein digests allowed the determination of the yields of those singly labeled cytochromes *c* that could not be separated by cation-exchange chromatography. The Y3 and Y4 fractions in one experiment and C2 and C3 in another were digested together and the two $[Pt(trpy)His]^{2+}$ chromophores detected by monitoring at 342 nm. Since both peptides are singly labeled, the ratio of their peak areas equals the ratio of the two singly labeled cytochromes in the mixture. The findings are included in Table III. The derivative C4 yielded equimolar amounts of two labeled peptides, whose retention times were identical with those of the peptides obtained separately from the C2 and C3 derivatives. The two binding sites in the doubly labeled *C. krusei* protein (C4) evidently are the same as the sites in the two singly labeled proteins (C2 and C3).

The lack of resolution of C2 and C3 and of Y3 and Y4 may well be a consequence of the similarity between His-33 and His-39. The overall charge of the protein is the same whichever residue is tagged. Even the charge distribution probably does not depend much on the binding site because the two residues are relatively close to each other on the protein surface.

V. Low Reactivity of Cys-102

Evidence. Four findings prove that the yeast protein is labeled primarily at His residues. (1) The difference spectra of the kind shown in Figure 2, obtained with the major labeled derivatives, are conclusive. Even when the incubation is carried out at pH 8.6, at which the highly nucleophilic thiolate form predominates over the thiol form, His-labeled derivatives remain the major ones. (2) The large difference in the rates with which imidazole and thiolate ligands displace the Cl^- ion from $[Pt(trpy)Cl]^+$ (see Table II) permits a kinetic proof of the major binding sites. The rate of protein tagging is identical, within the margin of experimental error, with the average rate of the reactions involving imidazole-containing ligands. The horse protein, which does not contain free Cys and is labeled only at His residues, reacts at this rate. (3) A mixture of the labeled derivatives of yeast cytochrome *c*

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behaves like the native protein when it gives a dimeric protein held by a disulfide bond. Size-exclusion chromatography of the mixture oxidized with air in the presence of Cu^{2+} ions yielded a very small amount of the monomeric protein, thus proving that Cys-102 is labeled sparingly. The dimer of the native protein and the dimer of the labeled protein derivatives are eluted from the size-exclusion column with the same retention time, 1.60 times the void time. The $Pt(trpy)^{2+}$ chromophores on the protein dimer are evident in the UV–vis spectrum—the proof that the tags were not displaced from the Cys 102 residues prior to the coupling of the protein molecules. (4) Carboxymethylation of Cys 102 with iodoacetamide (under the conditions at which this reagent does not block His residues) prior to the incubation with $[Pt(trpy)Cl]^+$ prevents the formation of derivatives Y2, Y5, and Y6, but does not affect the yields of other derivatives. This finding confirms Cys-102 as the single labeled residue in Y2 and as one of two such residues in the derivatives Y5 and Y6. It also confirms His-33 and His-39 as the labeled residues in the doubly labeled derivative Y7. Peptide mapping experiments with derivatives Y3 and Y4 proved that Y2 is not admixed to them. No $Pt(trpy)^{2+}$-labeled peptide was eluted at 52 min, the retention time of the labeled peptide T20 from derivative Y2.

Explanation. The low reactivity of the Cys residue and reactivity of the His residues toward the platinum complex are surprising in view of several facts. Relative nucleophilicities of the two potential ligands,[9,20] manifest in the outcomes of the reactions in eq 1 and 2 and in the complete selectivity of $[Pt(trpy)Cl]^+$ toward γ-Glu-*Cys*-Gly over Gly-*His*-Gly, predict the opposite. Residue Cys-102 is modified with various organic reagents.[12-18] Our whole experience with $[Pt(trpy)Cl]^+$ indicates that the yield of its reaction with a given ligand in the protein is proportional to the exposure of that ligand on the protein surface. The low reactivity of Cys-102, therefore, means that this residue is inaccessible. If it were accessible, it would have been labeled rapidly and completely. If it were somewhat accessible, its partial labeling would have raised the overall rate of the reaction between the yeast protein and $[Pt(trpy)Cl]^+$. The low reactivity of $[Pt(trpy)Cl]^+$ toward Cys-102 indeed demonstrates the noninvasiveness of this new labeling reagent. We conclude, in spite of the general assumption to the contrary,[16] that Cys-102 must be inaccessible from solution and that the reagents that modify Cys-102 completely have to perturb the protein in a way that enhances the exposure of this residue. The commonly used iodoacetamide may well be such an invasive reagent. Indeed, the treatment of the carboxymethylated yeast protein with $[Pt(trpy)Cl]^+$ under the usual conditions results in denaturation of ca. 50% of the protein. The partial unfolding of the C-terminal helix, necessary for the carboxymethylation of Cys-102, destabilizes the protein structure and perhaps renders His-18, an axial ligand to the heme, accessible to the platinum reagent.

Controlled partial unfolding of the *C. krusei* protein in the phosphate buffer containing 30% of methanol,[52] followed by incubation with $[Pt(trpy)Cl]^+$, caused an increase in the yields of labeling at His-33 and His-39. Similar preliminary experiments with the yeast protein showed that Cys-102 remains a minor binding site even in the presence of methanol.

These two conclusions—inaccessibility of Cys-102 and invasiveness of the reagents that modify it with large yields—were reached without knowledge of the protein structure. They were confirmed by the preliminary crystallographic analysis of the *iso*-1 cytochrome *c*, which was communicated to us afterward. Cysteine-102 indeed is buried in a hydrophobic region of the protein and inaccessible to the solvent.[48] Modifications with organic reagents and dimerization indeed are achieved by partial unfolding of the helical segment at the C terminus.[48] The new reagent, $[Pt(trpy)Cl]^+$, evidently does not perturb the protein in this way nor does it alter the structural and redox properties of the protein when it modifies the His residues on the surface.

VI. Advantages of the New Labeling Reagent

Complex $[Pt(trpy)Cl]^+$ is well suited to tagging biological macromolecules on account of its reactivity under very mild conditions and of its noninvasiveness, which permits selectivity toward exposed residues. An interplay between the steric and electronic properties of the terpyridyl ligand makes the selectivity of this complex opposite from that of the common reagent, $PtCl_4^{2-}$.[8] 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.[8]

A particular advantage of the new reagent lies in the strong UV–vis absorption by the $[Pt(trpy)L]^{n+}$ chromophore (extinction coefficients of 9000–30 000 M^{-1} cm^{-1}), which permits its easy detection and quantitation. The charge-transfer bands in the region 300–350 nm, largely unobscured by the protein absorption, are sensitive not only to the identity of the binding site (e.g., His vs Cys) but also to its environment.

The new inorganic reagent, unlike many well-known organic ones, does not perturb the protein to which it binds. Because of this important property, $[Pt(trpy)Cl]^+$ may perhaps be used as a reliable probe of the exposure of various binding sites on the protein surface. This study showed that $[Pt(trpy)Cl]^+$ exhibits great preference for cysteine and its peptides over histidine and its peptides but is nevertheless capable of labeling histidine residues in high yield in a protein that contains a reactive Cys residue. The seeming reversal of selectivity between amino acids and peptides on the one side and the yeast protein on the other is actually a consequence of the noninvasiveness of the novel reagent, which does not alter the protein conformation. The contrast between the molecules without and with tertiary structure may serve as a reminder that what is true about small models need not be true about biological macromolecules.

VII. Experiments

Materials and Methods. Iodoacetamide, (*p*-hydroxymercurio)benzenesulfonic acid, amino acids, peptides, and cytochromes *c* from *C. krusei* (of type VII), bakers' yeast (of type VIII-B), and horse heart (of type VI), were obtained from Sigma Chemical Co. The bakers' yeast protein contained less than ca. 5% of the *iso*-2 form. Chloro(2,2′:6′,2″-terpyridine)platinum(II) chloride dihydrate, $[Pt(trpy)Cl]Cl{\cdot}2H_2O$,[53] and (2-hydroxyethanethiolato)(2,2′:6′,2″-terpyridine)platinum(II) nitrate, $[Pt(trpy)(SCH_2CH_2OH)]NO_3$,[54] were obtained from Strem Chemicals. Oxidant $[Co(phen)_3](ClO_4)_3$ was prepared readily.[55] Deuteriated compounds were from Aldrich Chemicals. All dialyses were done by ultrafiltration with Amicon YM-5 membranes under nitrogen pressure at 4 °C.

The ^1H NMR spectra were recorded with a Nicolet NT 300 spectrometer at 22 °C. The protein samples were dialyzed, lyophilized repeatedly, and dissolved, all in D_2O. The residual water signal, at 4.79 ppm versus DSS, was used as an internal reference. Absorption spectra were recorded with an IBM 9430 UV–vis spectrophotometer, equipped with a two-grating monochromator. The difference spectra were obtained by subtraction of the spectra of fully oxidized protein samples from each other. The concentrations of the samples were equalized by matching of their Soret peaks at 410 nm. The X-band EPR spectra at 8 K were obtained with a Bruker 200D instrument. Cyclic and differential pulse voltammograms were recorded with an IBM EC 225 voltammetric analyzer, a BAS cell assembly, and an Ag/AgCl reference electrode. Molecular masses of the monomeric and dimeric protein from yeast were determined by size-exclusion chromatography on a column of Sephadex G 75-50, as detailed in another report from this laboratory.[56]

Amino Acid and Peptide Complexes $[Pt(trpy)L]^{n+}$. The reactions shown in Scheme I were effected by addition of 1.0 mL of a 5.0 mM solution of the ligand L to 1.0 mL of 5.0 mM solution of $[Pt(trpy)Cl]Cl$ and by incubation of the mixture for 1 day. Both water and 85 mM phosphate buffer at pH 7.0 were used as solvents. The reaction of $[Pt(trpy)Cl]^+$ with glutathione in 0.1% CF_3COOH was done similarly.

The reactions shown in eq 1 and 2 were effected, and those in eqs 3–5 were attempted, by mixing together equimolar amounts of the two reactants at room temperature; in a typical experiment, 1.0 mL of a 5.0 mM solution of each reactant in the 100 mM phosphate buffer at pH 7.0 was used. Compound $CysHgC_6H_4SO_3H$ was prepared by treatment of cysteine (250 μL of a 5.0 mM solution, 1.3 μmol) with *p*-

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$HOHgC_6H_4SO_3H$ (5.0 mg, 13 μmol) for 2 h; reaction 2 was then effected by addition of 200 μL of a 5.0 mM solution of $[Pt(trpy)Cl]^+$ to the mixture. Reactions 1 and 2 caused a color change from yellow to red in seconds, whereas reactions 3–5 failed to cause any color change even after three days.

Kinetic Measurements. The results are given in Table II. The relatively slow reactions with the three imidazole-containing ligands, shown in Scheme I, were followed by a Varian Cary 19 spectrophotometer. The relatively fast reactions with the three thiol-containing ligands, also shown in Scheme I, were monitored with an IBM 9430 spectrophotometer equipped with a thermostated rapid-kinetics accessory SFA-11 from Hi-Tech Scientific. All the solutions were made in 85 mM phosphate buffer at pH 7.0, thermostated at 25 °C, and the reactions monitored at 343 nm. Pseudo-first-order conditions were achieved with 20 μM $[Pt(trpy)Cl]^+$ and 2 mM entering ligands. The observed rate constants, k_{obsd}, were calculated from the absorbances by a nonlinear least-squares method.

Protein Labeling. Unless stated otherwise, all the incubations and chromatographic separations were done at 4 °C, with phosphate buffer at pH 7.0 as a solvent and an eluent. The bakers' yeast cytochrome *c* was treated with a tenfold excess of dithiothreitol (DTT) to ensure that it is entirely in the monomeric form,[13,57] and the organic reagent was dialyzed away. Ferricytochrome *c* from horse, *C krusei*, and yeast were incubated for 1 day with an equimolar amount of $[Pt(trpy)Cl]Cl$ (2 mM each) in 100 mM buffer. Some reactions with the yeast protein were done at pH 8.6. The reaction was terminated by dialysis into the same buffer, the protein was oxidized with 1 equiv of $[Co(phen)_3](ClO_4)_3$, and the excess oxidant was removed by dialysis. With all of the protein in the ferric state, cation-exchange chromatography was carried out on 15 cm × 1 cm columns of CM 52, equilibrated with 100 mM buffer. The typical sample contained 5 mg of cytochrome *c* in 0.5 mL of the same buffer. Regardless of the incubation conditions, the elution was done with the phosphate buffer at pH 7.0. The chromatographic results are summarized in Table III. The horse protein was chromatographed as in our previous study.[8] With both the *C. krusei* and yeast proteins, the singly labeled derivatives were eluted with 100 mM buffer at 5 mL h^{-1} but the doubly labeled ones required a 0.05 M solution of NaCl in this buffer and an elution rate of 20 mL h^{-1}.

Cysteine-102 in the yeast cytochrome *c* was carboxymethylated with iodoacetamide by known procedures.[13,58] The protein was treated with DTT in 85 mM Tris–HCl buffer at pH 8.6, as before. After dialysis, the protein was brought to a concentration of 200 μM. A solution of iodoacetamide, containing 0.3 mg of this chemical per 1.0 mg of the protein, was prepared in a volume of water equal to the volume of the buffered protein solution. The mixture of the two solutions was left at room temperature, in the dark, under a stream of nitrogen, for 6 h. Subsequent incubation with $[Pt(trpy)Cl]^+$ was done as before.

Dimerization of the yeast cytochrome *c* was achieved by the standard procedure.[12,14] The protein first was incubated with 1 equiv of $[Pt(trpy)Cl]^+$ as usual. After the dialysis of the unbound $[Pt(trpy)Cl]^+$, the mixture of the native protein and its $Pt(trpy)^{2+}$-tagged derivatives was incubated with $CuSO_4$ in the presence of air. The $CuSO_4$ was dialyzed away, and the molecular mass of the protein was determined by size-exclusion chromatography.

Peptide Mapping. The published procedures[59] were modified in a minor way. The bakers' yeast cytochrome *c* and its derivatives were treated with *p*-$HOHgC_6H_4SO_3H$ before hydrolysis. To 5.0 mL of a 200 μM solution of the protein in 100 mM phosphate buffer at pH 7.0 was added 1.0 mL of 1.0 mM solution of the organomercurial reagent in the same buffer. After 2 h, the reagent was dialyzed away and the protein transferred into water by ultrafiltration. Tryptic hydrolysis was done at pH 7.0 in water. The protein samples were brought to a concentration of 20–200 μM and treated with 50 μL of the solution containing 2 mg of trypsin in 1 mL of 4 mM HCl. Another aliquot of trypsin was added after 6 h, and the digestion was ended after 10 h by lyophilization. The digest was dissolved in the starting buffer (49 mM KH_2PO_4 and 5.4 mM H_3PO_4) and subjected to HPLC separation with a linear gradient between the buffer and acetonitrile; the fraction of the latter component increased from 0 to 45% in 2 h. The $Pt(trpy)^{2+}$-tagged peptides were detected at 342 nm and identified by their retention times or by their composition, or by both properties.

Samples for amino acid analysis were hydrolyzed at 165 °C for 45 min under argon; they were then dried and diluted with water of HPLC grade. The derivatives were prepared and separated with an Applied Biosystem 420A instrument. The quantities of amino acids were calculated with a Model 920A module.

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