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Selective Labeling, Cross-Linking, and Cleavage of Proteins with Transition-Metal Complexes

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Selective Labeling, Cross-Linking, and Cleavage of Proteins with Transition-Metal Complexes

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Because the substitution reactions of transition-metal complexes can be controlled precisely by the choice of the metal and the ligands, and because these complexes have various spectroscopic and electrochemical properties, they are well-suited for many applications in biochemical and biophysical laboratories. Complex $[Pt(trpy)Cl]^+$ reacts, under mild conditions and noninvasively, with the exposed side chains of His, Cys, and even Arg residues in proteins. The derivatives can be separated chromatographically. The $Pt(trpy)^{2+}$ tags are stable. They are easily detected and quantitated owing to the strong UV-vis bands, whose positions and relative intensities depend on the identity and the environment of the tagged residue. The selectivity of Pt(II) chloro complexes as labeling reagents can be controlled by purposeful changes of the other three ancillary ligands. Treatment of Cys residues with $(\eta^5\text{-}C_5H_5)Fe(\eta^5\text{-}C_5H_4HgCl)$ results in attachment of the redox-active ferrocenyl tag to the protein. Proteins can be cross-linked covalently through Met residues with the monometallic *trans*-PtL_2Cl_2 reagents, and through His residues with the bimetallic $Rh_2(RCOO)_4$ reagents. Cross-linking with the trimetallic reagent $[Ru_3(\mu_3\text{-}O)(\mu_2\text{-}OAc)_6(H_2O)_3]$ may yield covalent triprotein clusters. A $PtCl_3^-$ group attached to the Cys side chain in glutathione promotes selective hydrolysis of the Cys–Gly peptide bond. These findings point the way for the study of protein topography, of enzyme mechanism, of electron-transfer reactions in modified metalloproteins, of metal binding to Arg residues, and of protein cleavage by inorganic reagents.

Key Words: *covalent modification, affinity labeling, electron transfer, metalloproteins, platinum, peptide hydrolysis*

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INTRODUCTION

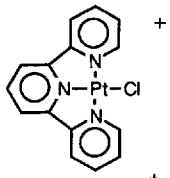
Covalent modification of proteins, cross-linking of proteins to one another and to other biomolecules and membranes, and hydrolysis of peptide bonds are some of the principal biochemical procedures. The labeling and cross-linking is commonly done with organic reagents,[1] and hydrolysis with proteolytic enzymes. Except as heavy-atom scatterers in crystallographic studies,[2,3] metal complexes have not been used widely for any of the aforementioned three purposes.

A good reagent for labeling or cross-linking must meet various requirements. It should be sufficiently inert toward hydrolysis; reactive under mild conditions; selective toward particular amino-acid side chains; easy to detect and quantitate in the modified protein; stable, yet removable, so that the native protein can be restored; and noninvasive, so that the structure and function of the modified protein are not altered.

Examples discussed in this article show that transition-metal complexes can have the required properties. In order to achieve selective attachment to amino-acid side chains, preformed complexes rather than the "bare" metal ions should be used. The reactivity, stability, and detectability of the metal-containing tags and cross-links can then be achieved by the purposeful choice of the metal atoms and of the reactive and ancillary ligands. Although there are several successful approaches to the labeling of proteins with metal complexes, the length of this article permits only a review of the work in the author's laboratory. This work has involved stable, well-studied proteins (several cytochromes *c*, papain, and chymotrypsin) and also amino acids, their derivatives, and peptides.

LABELING OF HISTIDINE AND CYSTEINE IN CYTOCHROMES *c*[4,5]

Chloro(2,2′:6′,2″-terpyridine)platinum(II), shown below, has been used before as an intercalation agent for DNA.[6] But it is also well-suited for covalent binding to biomolecules, especially to amino-acid side chains in proteins. Because only the chloride ion can be



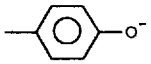
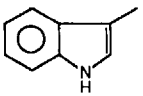
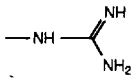
[Pt(trpy)Cl]$^+$

displaced from the complex under ordinary conditions, this reagent can form only 1:1 adducts. Because complexes of Pt(II) are kinetically inert, the $Pt(trpy)^{2+}$ tag is stable on the protein. Because the tag is a UV-vis chromophore, it is easily detected and quantitated. All the reactions were carried out with $[Pt(trpy)Cl]Cl{\cdot}2H_2O$, which is prepared easily[7a]; it is sold by Aldrich Chemical Co.[7b]

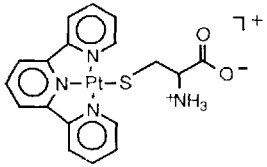
The potential ligands in proteins are mainly the heteroatoms in those side chains shown in Table I. The corresponding amino acids,

TABLE I

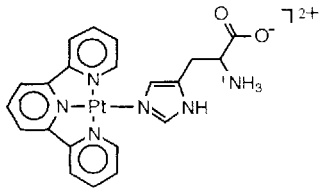
Potential ligands for transition metals in proteins

Group	Typical pK	Amino Acid
$-COO^-$	4.0	Asp, Glu
$-C(O)NH_2$		Asn, Gln
	6.5	His
$-S^-$	8.5	Cys
$-S-CH_3$		Cys–Cys
	10.0	Tyr
$-NH_2$	10.5	Lys
		Trp
	12.5	Arg

and some peptides containing them, were incubated at room temperature with the equimolar amount of $[Pt(trpy)Cl]^+$ complex under the conditions used later for protein modification. The following amino acids caused no change in the UV-vis spectrum: lysine, tryptophan, aspartic acid, asparagine, glutamic acid, glutamine, proline, threonine, serine, tyrosine, cystine, and methionine. (Arginine reacted upon standing or heating, as will be detailed below.) Cysteine, homocysteine, and reduced glutathione (GSH) reacted rapidly because of the high nucleophilicity of the thiolate ligands toward the Pt(II) atom. Imidazole (Im), histidine, and the tripeptide Gly–His–Gly reacted slower because of the lesser nucleophilicity of these nitrogen ligands toward Pt(II). The rate constant for the thiol-containing ligands is approximately 300 times greater than that for the imidazole-containing ones. Because of this large kinetic difference, the most reactive amino acid in proteins should be free cysteine, and the next one in reactivity should be histidine. The corresponding complexes are shown below.



$[Pt(trpy)Cys]^+$



$[Pt(trpy)His]^{2+}$

The unreactivity of methionine, cystine (Cys–Cys), and oxidized glutathione (GSSG) was surprising in view of the high affinity of the soft Pt(II) complexes toward the soft thio-ether and disulfide ligands. Persistent attempts with methionine and its various derivatives and peptides proved their complete inertness toward $[Pt(trpy)Cl]^+$ even under forcing conditions. Molecular models of the pentacoordinate $[Pt(trpy)ClL]^+$ molecules, representing the intermediate or the transition state in the non-occurring reaction with the thio-ether and disulfide ligands L, showed no prohibitive crowding. But models of the expected product, $[Pt(trpy)L]^{2+}$, showed such crowding. Nonparametrized molecular orbital calculations on $[Pt(trpy)SMe_2]^{2+}$, which mimics the expected complex with methionine, revealed a strong repulsion between a methyl

group in SMe_2 and an ortho H atom in the cis pyridine ring. This interaction could not be avoided by rotation of the pyramidal thio-ether ligand about the Pt–S bond. Whereas the complex $[Pt(trpy)SMe_2]^{2+}$ seemed inherently unstable, the complex $[Pt(trpy)Im]^{2+}$ seemed devoid of steric repulsions and stable. This contrast is a quantum-mechanical explanation of the unexpectedly different behavior of methionine and histidine toward $[Pt(trpy)Cl]^+$.

Labeling of each cytochrome *c* was accomplished simply, under mild conditions. The protein was incubated with an equimolar amount of $[Pt(trpy)Cl]^+$, in phosphate buffer at pH 5.0 or 7.0, for about one day. The reaction mixture was then chromatographed efficiently on CM 52 cation exchanger, with phosphate buffer as an eluent. The greater the number of the cationic $Pt(trpy)^{2+}$ tags on the protein, the slower its movement down the column. Singly, doubly, and triply labeled derivatives—[Pt(trpy)cyt], $[\{Pt(trpy)\}_2cyt]$, and $[\{Pt(trpy)\}_3cyt]$, respectively—were eluted in this order. (The overall charge of protein-containing complexes is not specified because it is variable.) Although the derivatives that are singly labeled at differently located residues of the same kind have the same overall charge, they were separated owing to their different distributions of charge. The $Pt(trpy)^{2+}$ tags survived the cation-exchange chromatography because the ligands on the protein formed inert complexes with the Pt(II) atoms.

The inorganic chromophores were clearly evident in the UV-vis spectra of the tagged protein because, as Fig. 1 shows, their bands were largely unobscured by those of the protein itself. Systematic subtractions of the spectra of the native and modified proteins from one another produced the spectra of the $Pt(trpy)^{2+}$ tags. The nonspecific absorptions of the aromatic trpy ligand in the region 240–280 nm (ϵ = 19,000–31,000 M^{-1} cm^{-1}) proved useful because of their intensity. The absorption patterns in the region 320–350 nm were weaker (ϵ = 9,000–16,000 M^{-1} cm^{-1}), but characteristic of the ligand L in $[Pt(trpy)L]^{n+}$. Matching of the difference spectra with the spectra of model complexes containing amino acids and peptides as L allowed both counting of the tags and identification of the types of the tagged amino acids in the protein derivatives. Not only do the band positions depend on the nature of the ligand L (i.e., histidine vs. cysteine), their relative intensities depend on the environment of this ligand. For example, [Pt(trpy)

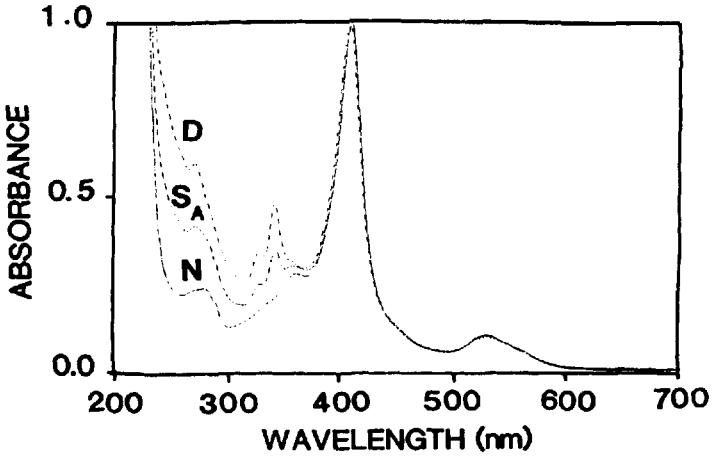


FIGURE 1 Electronic absorption spectra of the native (N) horse-heart cytochrome *c* and of its derivatives designated S_A and D. The former is singly labeled with a $Pt(trpy)^{2+}$ chromophore at the site marked A (His 33), whereas the latter is doubly labeled at sites marked A (His 33) and B (His 26). (Reproduced with permission of the American Chemical Society, from Ref. 3.)

His]$^{2+}$ tags at His 33, in the hydrophilic region, and at His 26, in the hydrophobic region, both exhibited the characteristic bands at 342 and 328 nm, but the relative intensities of these bands were different. The $[Pt(trpy)Cys]^+$ tag exhibited also a third band, at 311 nm.

Various methods were used to determine the binding sites in the cytochromes *c*: peptide mapping; comparisons among the derivatives obtained from the proteins with slightly different sequences; selective blocking, prior to labeling with $[Pt(trpy)Cl]^+$, of certain residues by protonation or by covalent modification; and measurements of the rates at which the proteins took up the inorganic chromophore. The labeled amino-acid residues are listed in Table II. The yields of the platinated derivatives are approximately proportional to the exposure of the respective residues on the protein surface, as determined by X-ray crystallography.[8,9]

The inorganic labels do not perturb the protein conformation, its redox potential, and the electronic and geometric structure of the active site. This general conclusion emerged from systematic

TABLE II

Residues in cytochrome *c* that are labeled in reactions with $[Pt(trpy)Cl]^{+}$[a]

Organism	His 26	His 33	His 39	Arg 91	Cys 102
Horse	√	√		√	
Tuna	√			√	
Bakers' Yeast	√	√	√	–	√
Candida krusei	√	√	√	–	

[a]Check, labeled; dash, not labeled; blank, unreactive amino acid in that variant position in the sequence.

comparisons of the singly and multiply labeled protein derivatives with one another and with the native proteins. The following methods were used: UV-vis spectrophotometry, cyclic voltammetry, differential-pulse voltammetry, EPR spectroscopy, and ^1H NMR spectroscopy.

The relatively low reactivity of Cys 102 in the bakers'-yeast cytochrome *c*—the yield of the singly-labeled derivative was only ca. 10%—was surprising because this residue can be modified with organic reagents[10] and because the protein can dimerize readily through a disulfide bond.[11] The histidine residues 33 and 39 proved considerably more reactive—the corresponding yields were ca. 15 and 20%—even though the thiol ligands are far more nucleophilic than imidazole ligands toward $[Pt(trpy)Cl]^{+}$. The 300-fold difference between the rate constants for the two types of ligands, discussed above, permitted a kinetic proof of the binding group. The bakers'-yeast cytochrome *c* reacted with $[Pt(trpy)Cl]^{+}$ at the same rate as did its horse congener (which lacks free Cys), the tripeptide Gly–His–Gly, and other imidazole-containing ligands. This unexpected outcome of the protein labeling indicated 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 conformational change that makes Cys 102 in the bakers'-yeast cytochrome *c* accessible to the reagents and to another molecule of this protein. These predictions from the labeling study were confirmed subsequently by the crystallographic analysis of this cytochrome *c*; indeed, Cys 102 points toward the protein interior.[12]

Verification of the binding sites in the bakers'-yeast protein by peptide mapping required special precautions to ensure that the $Pt(trpy)^{2+}$ tags remained at the points of initial attachment. Experiments with model amino-acid complexes showed that the tag can migrate from histidine to free cysteine, but not to mercurated cysteine. Although Cys 102 is almost inaccessible in the native protein, it becomes fully exposed in the protein digest. A standard procedure—enzymatic cleavage of the protein tagged at His residues—would have been misleading; the tag would have migrated readily and would have been detected on the peptide containing Cys 102 rather than on those containing His 33 and His 39. Prior to tryptic digestion, therefore, the free Cys 102 in the $Pt(trpy)^{2+}$-modified protein was blocked with $HOHgC_6H_4SO_3^-$, and the peptide mapping became reliable.

The complex $[Pt(trpy)Cl]^+$ differs from some of the standard labeling reagents by its noninvasiveness: the ease of its attachment to a ligand of a certain type reflects this ligand's true exposure on the protein surface. This property may well render the new inorganic reagent useful as a probe of the protein topography and applicable even to proteins whose structures are unknown or only partially known.

LABELING OF HISTIDINE AND CYSTEINE IN PROTEOLYTIC ENZYMES[13]

Experiments with papain showed that selectivity and high yield in labeling with metal complexes can be achieved by exploiting the differences in nucleophilicities among the amino-acid side chains and common inorganic ligands. The active site of this protease contains exposed residues Cys 25 and His 159, which are essential to the catalytic action. Because the thiol group has much higher affinity than imidazole toward Pt(II), incubation of the enzyme with an equimolar amount of $[Pt(trpy)Cl]^+$ resulted in the labeling of the former, although with a partial yield. The full labeling of Cys 25 was achieved by the following procedure: incubation with a 10-fold excess of $[Pt(trpy)Cl]^+$, dialysis, and incubation with a 10-fold excess of KI. The first treatment resulted in full labeling of both residues. The next treatment removed the tag only from His 159 because the I^- anion is more nucleophilic than imidazole,

but far less nucleophilic than thiolate. The well-known relative nucleophilicities of ligands toward Pt(II)[14] permit, in principle, preparation of multiply labeled derivatives from one another by selective and sequential removal of the platinum tags from the amino-acid side chain in this order: histidine, methionine, cysteine. The conditions for the stepwise removal can be determined in systematic studies of the rates and equilibria of displacement reactions involving platinum complexes with amino acids, peptides, and various inorganic ligands.

Studies of chymotrypsin labeled with the new reagent have revealed some unexpected properties of this protease.[13] It is similar to papain, but has Ser 195 instead of the cysteine at the active site. Incubation with $[Pt(trpy)Cl]^+$ resulted in selective labeling of just two residues: His 57 at the active site and His 40 elsewhere on the protein surface. The former is essential for the activity, and its labeling with so-called TPCK inactivates the enzyme. When, however, this residue (as well as His 40) was labeled with $Pt(trpy)^{2+}$, the enzyme retained ca. 1% of its activity toward *p*-nitrophenylacetate (an ester) and toward N-benzoylarginine-*p*-nitroanilide (an amide). These surprising findings throw fresh light on the mechanism of action of this well-known enzyme. Perhaps TPCK and $[Pt(trpy)Cl]^+$ differently affect the activity because they differently recognize His 57 as the binding site. The organic reagent is selective by dint of its analogy with the substrate and of its consequent affinity for the entire active site, whereas the inorganic reagent is guided by its affinity just for the imidazole ligand. Perhaps in the former case the active site is filled, whereas in the latter it may still be accessible to the substrate.

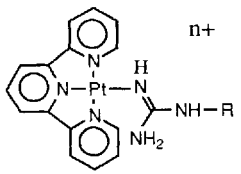
The $Pt(trpy)^{2+}$ chromophore at His 57 apparently senses its environment within the active site. The absorptivity quotient $\epsilon_{341}/\epsilon_{327}$ changed with pH, and an inflexion in the plot occurred around pH 3.0. Since the pK value of the Asp 102 side chain is 2.8, it may well be the charge of this proximate residue, which is essential to the enzyme function, that affected the chromophore.

LABELING OF ARGININE AND METAL–GUANIDINE COMPLEXES[15,16]

The recognized functions of Arg residues in proteins—binding of cofactors and anions—involve electrostatic attraction to the guan-

idinium cation. Although transition metals are common in proteins, their covalent binding to Arg side chain has not been proposed. Indeed, metal–guanidyl complexes are barely known.

When cytochromes *c* from horse and tuna were incubated with $[Pt(trpy)Cl]^+$ at pH 7.0 (rather than 5.0) and chromatographed as before, additional $Pt(trpy)^{2+}$ derivatives were obtained. Their number and their difference UV-vis spectra indicated that the increase in pH had created a single new binding site, which is the same in all the new derivatives and is not a histidine. This site was identified indirectly, but conclusively, by monitoring of reactions between $[Pt(trpy)Cl]^+$ and amino acids, and by considerations of the cytochrome *c* structures. The new ligand must be accessible in both horse and tuna proteins, and there must be few such ligands because only one reacts. Only three residues—Tyr 74, Tyr 97, and Arg 91—satisfy both requirements. Thorough tests, with heating, confirmed the unreactivity of Tyr and reactivity of Arg. N-Acetylarginine (AcArg) and such simple ligands as methylguanidine (MeGua) and guanidine (Gua) yielded homogeneous complexes, shown below.



R	n	L
H	2	Gua
CH_3	2	MeGua
$(CH_2)_3CH(NH_3)(COO)$	2	Arg
$(CH_2)_3CH[CH_3C(O)NH](COO)^-$	1	AcArg
$O(CH)_2)_2CH(NH_3)(COO)$	2	Can

Their UV-vis spectra were very similar to the difference spectra of those labeled proteins that correspond to the $Pt(trpy)^{2+}$ tag at the new site. Evidently, this site is Arg 91.

Why the side chain of Arg 91 reacts at pH 7.0 despite the guanidine basicity—its normal pK value is 12.5—became evident from the structure of cytochrome *c*.[8,9] Barely exposed on the surface (hence its low labeling yield of ca. 10%), Arg 91 abuts the N-terminus of the α-helical segment 92–102. The macrodipole of this segment and the hydrophobic environment together lessen the basicity of Arg,[17] so it reacts with $[Pt(trpy)Cl]^+$ even in neutral solution. This explanation was confirmed in experiments with canavanine (Can), a close homolog of arginine whose guanidine group

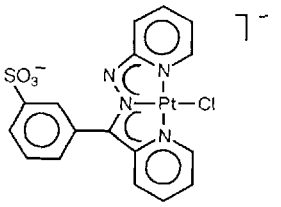
has a pK value of 7.0. Canavanine formed the $[Pt(trpy)Can]^{2+}$ complex readily under the conditions of the cytochrome labeling.[16]

This evidence of Pt(II) binding to arginine in proteins, together with a previous report of the bioactive metals Cu(II), Co(II), Zn(II), and Cr(III) binding to tetramethylguanidine,[18] demonstrates that arginine side chain is a potential ligand in metalloproteins and in metal-dependent enzymes. Arginine coordination should be facilitated by environmental effects, such as hydrophobicity and proximity to α-helical polypeptide segments, that reduce the basicity of the guanidine group. Since the metal–guanidine bonding probably involves some π back donation from the metal to the trigonal N atom, this bonding may be facilitated by π donation to the metal atom from other ligands, such as thiolate or phenoxide. Therefore arginine is likely to coordinate to transition metals in conjunction with cysteine and tyrosine ligands. Although arginine certainly is not as common a bioligand as histidine, the similarity between the trigonal N atom in the guanidine group and the pyridine-type N atom in the imidazole ring is worth noting.

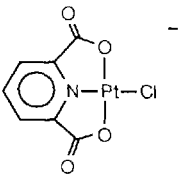
ANCILLARY LIGANDS AND SELECTIVITY

The unreactivity of $[Pt(trpy)Cl]^+$ toward methionine and its reactivity toward histidine are consequences of an interplay between steric and electronic properties of the terpyridine ligand. The ortho H atoms repel the thio ether, otherwise a very nucelophilic ligand. The aromatic character of the complex, however, labilizes the chloride ligand,[19] and facilitates its displacement by the imidazole, otherwise a less nucleophilic ligand toward Pt(II).[14] The decisive effect of the chelate ligand on the selectivity is evident from the comparison between $[Pt(trpy)Cl]^+$ and $[PtCl_4]^{2-}$. Although both of them are Pt(II) chloro complexes, the second one exhibits such a large preference for methionine over histidine in proteins that it is used as a methionine-specific heavy-atom tag in protein crystallography.[20] Indeed, our molecular orbital calculations revealed no steric crowding in $[PtCl_3(SMe_2)]^-$ and in $[PtCl_3Im]^-$ because the Cl^- ligands are sufficiently small. In the absence of steric effects, evidently, the displacement reactivity is governed by the nucleophilicity of the entering ligands.

The four Pt(II) complexes discussed below all have the same displaceable ligand, Cl^-, but differ from one another in the ancillary ligands and, consequently, in charge. All these complexes except the Zeise's anion were synthesized purposefully in our laboratory. Although the studies of their reactivity toward cytochromes *c* were not as thorough as studies involving $[Pt(trpy)Cl]^+$, certain interesting differences emerged.



$[Pt(sbpaphy)Cl]^-$

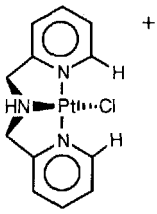


$[Pt(dipic)Cl]^-$

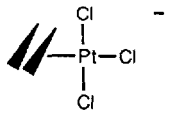
The tridentate ligand 2-(3-sulfobenzoyl)pyridine-2-aldehyde 2′-pyridylhydrazone, designated sbpaphy, is similar to terpyridine for it contains three coplanar trigonal nitrogen atoms, but it coordinates to metals as a dianion. The difference in charge between $[Pt(sbpaphyCl]^-$ and $[Pt(trpy)Cl]^+$ did not alter the binding selectivity—the former complex still labeled His 33 in the horse cytochrome *c*—but did change the mobility on the cation-exchange column. Although the Pt(sbpaphy) tag as a whole is neutral, the modified protein eluted before the native one, perhaps because of the electrostatic repulsion between the sulfonate group in the tag and the carboxylate groups of the CM 52 cation exchanger.[21]

Dipicolinate dianion (dipic) binds as a tridentate ligand in $[Pt(dipic)Cl]^-$, but its carboxylate "arms" are sterically undemanding in comparison with the terminal pyridine rings of terpyridine.[22] Because of this reversal, the new complex (as a K^+ salt) reacted with methionine faster than with histidine. Incubation with horse cytochrome *c* yielded a single derivative, which probably is labeled at Met 65. Binding to Met 80, an axial ligand to the iron atom, is ruled out because the spectroscopic and redox properties of the heme were unperturbed.[23] The new Pt(II) complex, which is not sterically congested, can react with the thio-ether ligand, the most nucleophilic one in the horse cytochrome *c*.

Ligand 2,2′-dipicolylamine (dpa) is akin to terpyridine except that the central N atom is tetrahedral rather than trigonal, and that the terminal pyridyl rings are connected with an aliphatic tether rather than with a third aromatic ring. A model of the $[Pt(dpa)Cl]^+$ complex shows an important consequence of the pyramidality of the central N atom. Although the PtN_3Cl skeleton is still planar, the chloride ligand lies away from the $Ptpy_2$ plane and is therefore less shielded by the ortho hydrogen atoms, which lie in this plane. Experiments with cytochrome *c* will show whether this moderate alleviation of the steric hindrance will alter the selectivity and permit the new reagent to label Met 65.[23]



$[Pt(dpa)Cl]^+$



$[Pt(\eta^2\text{-}C_2H_4)Cl_3]^-$

The reagents discussed so far are coordination complexes, i.e., the donors in them are "inorganic" atoms. Experiments with Zeise's salt, $K[Pt(\eta^2\text{-}C_2H_4)Cl_3]$, showed that organometallic complexes, too, can be used for the labeling of proteins.[24] The reaction of horse cytochrome *c* with this salt is unlike the reactions with other labeling reagents. Incubation of the protein with variable excesses of $[Pt(\eta^2\text{-}C_2H_4)Cl_3]^-$, followed by ultrafiltration and by UV-vis determination of the Pt complex in the filtrate, showed that one molecule of the protein can consume as many as ten Zeise's anions in a relatively fast reaction. If the tags become covalently attached, the most likely ligands for them are the amine side chains of Lys residues, of which there are 19 and which are exposed on the protein surface. Indeed, Zeise's anion is known to easily form *trans*-$[Pt(\eta^2\text{-}C_2H_4)(RNH_2)Cl_2]$ complexes with primary amines.[25] Some uncertainties notwithstanding, the experiments with Zeise's anion showed the effect of the ethylene ligand, a potent trans-labilizer, upon the reaction with cytochrome *c*. The displacement of the Cl^- ligand became easier and the protein was labeled faster, but less selectively.

A REDOX-ACTIVE LABEL

The high affinity of organomercurials toward free Cys residues permits interesting modifications of proteins. Reaction of chloromercuryferrocene, designated FcHgCl, with the bakers'-yeast cytochrome *c* resulted in attachment of the ferrocenylmercury group to the Cys 102 residue according to Eq. (1).[26]

$$\text{CYT(Cys 102)–SH} + \text{FcHgCl} \longrightarrow \text{CYT(Cys 102)–S–HgFc} + \text{H}^+ + \text{Cl}^- \quad (1)$$

Incubation of the monomeric protein with an equimolar amount of FcHgCl, oxidation with $K_3[Fe(CN)_6]$, dialysis, and chromatography on CM 52 yielded the labeled momomeric protein and the unlabeled dimeric protein as well-separated fractions. Since the ferrocenyl group is redox active, it was detected easily by cyclic voltammetry and by differential-pulse voltammetry. The labeled protein showed a characteristic wave at 450 mV versus NHE; it was attributed to the FcHgCys complex, which is accessible to the electrode. Addition and removal (by dialysis) of 4,4′-bipyridine, a redox mediator, caused appearance and disappearance of a wave at 270 mV; it was attributed to the inaccessible heme. Studies of electron transfer between the iron atoms in the organometallic tag and in the protein are going on.

The attachment at Cys 102 was proved in two ways. First, the horse cytochrome *c*, whose sole qualitative difference from its bakers'-yeast congener is its lack of Cys 102, did not form stable adducts with FcHgCl. Second, the labeled bakers'-yeast protein did not undergo dimerization, a process that requires free Cys 102 for the formation of an interprotein disulfide bond.[11]

Although the reaction of FcHgCl with the bakers'-yeast cytochrome *c* is a substitution, the reaction of this organometallic compound with free cysteine is a disproportionation.[27] Our crystallographic study of diferrocenylmercury, obtained according to Eq. (2), revealed the expected trans (or anti) conformation of the two ferrocenyl moieties, linked linearly by a Hg atom.

$$2\,\text{CysH} + 2\text{FcHgCl} \longrightarrow \text{Fc}_2\text{Hg} + \text{Hg(Cys)}_2 + 2\,\text{H}^+ + 2\,\text{Cl}^- \quad (2)$$

Numerous studies of organomercurials and thiol compounds have stressed the substitution reactions, but our study with cysteine shows that disproportionation, too, must be taken into account. In view of the abundance of glutathione (γGlu–Cys–Gly) and of other thiols in the living cell, the disproportionation reactions may play a role in the toxicity of organomercurials.

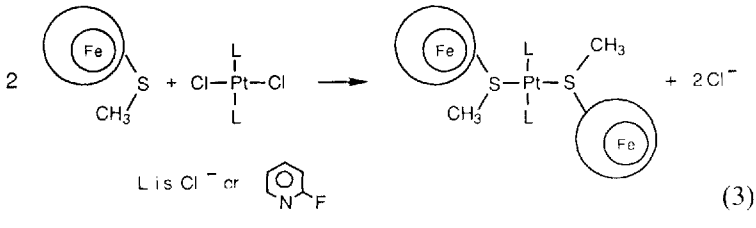
SELECTIVE CROSS-LINKING[28,29]

The cross-linking of proteins is commonly done with bifunctional organic reagents. The advantages of inorganic reagents, listed in the Introduction, can be exploited in this application, too.

Because only the chloride ion can be displaced easily from the tridentate complexes [Pt(chelate)Cl], only one protein molecule can bind to the Pt atom. More than one chloride ion can, however, be displaced from the unidentate complex $[PtCl_4]^{2-}$. Although crystallographers have commonly used this complex as a heavy-atom tag for proteins,[20] cross-linking has not been observed because the tagging has invariably been done with crystals, in which the protein molecules are immobile. Our research has shown for the first time that proteins, exemplified by cytochrome *c*, in solution can be cross-linked selectively, under mild conditions, with $[PtCl_4]^{2-}$ and with its derivatives.[28]

Incubation of horse cytochrome *c* with $K_2[PtCl_4]$ yielded a stable diprotein complex, *trans*-$[PtCl_2(cyt)_2]$, which was separated from the native and $PtCl_3^-$-tagged proteins by size-exclusion (gel-filtration) chromatography on Sephadex 75–50. The molecular mass of 30.7 ± 0.8 kDa, determined by this method, confirmed the composition of the diprotein complex. This value is slightly greater than expected, because the mobility of a macromolecule down the Sephadex column depends not only on the mass of the macromolecule, but also on its shape. The complex, which consists of two protein molecules bridged by a $PtCl_2$ group, appeared to the Sephadex gel slightly larger than a single spheroidal molecule of the same mass.

The binding group in each of the two protein ligands is Met 65, and the diprotein complex is stable owing to the affinity of the thioether group for the Pt(II) atom; see Eq. (3).



(3)

The complex was cleaved, however, by incubation with highly nucleophilic ligands, such as thiourea, which displaced the proteins and extruded the platinum link. This combination of stability under ordinary conditions and easy removability under conditions harmless to the protein is a particularly useful feature of the $PtCl_2$ link.

This link proved detectable by IR spectroscopy. A single IR band at 343 cm^{-1}, characteristic of the Pt(II)–Cl bond, indicated a *trans* configuration of the chloride ligands, and thus also of the protein molecules. This configuration was confirmed in experiments involving *cis*-$[Pt(bpy)(DMF)_2]^{2+}$, a complex containing two labile dimethylformamide ligands in cis positions to each other. One, but not both, of the DMF ligands proved displaceable with cytochrome *c*, evidence that two protein molecules cannot occupy cis positions in a Pt(II) complex. Even a cursory examination of molecular models confirmed this impossibility. The shortest distance between the two proteins in *trans*-$[PtCl_2(cyt)_2]$ is approximately 4.5–5.0 Å, comparable with the separation between the nearest points of two cytochrome *c* molecules in the crystalline unit cell. Cross-linking through a *trans*-$PtCl_2$ unit apparently does not force the protein molecules too close to each other. Spectroscopic and electrochemical measurements showed no significant perturbation of the geometric and electronic structure of the cytochrome *c* upon cross-linking.

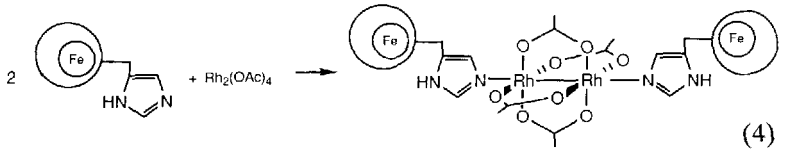
Since only two chloride ligands in $[PtCl_4]^{2-}$ are displaceable by the protein molecules, the other two ligands can be changed, and thus the properties of the linking reagent altered, in a purposeful way. Such an altered reagent is *trans*-$[Pt(2\text{-}Fpy)_2(DMF)_2]^{2+}$, whose incubation with the horse protein yielded *trans*-$[Pt(2\text{-}Fpy)_2(cyt)_2]$. The two 2-fluoropyridine ligands in the diprotein complex were observed by ^{19}F NMR spectroscopy and thus the detection of the

bridge between the protein molecules simplified. This variability of the ancillary ligands is yet another advantage of the new inorganic reagents for cross-linking of proteins.

The formation of the diprotein complexes, shown in Eq. (3), illustrates a classical concept in coordination chemistry. The first protein ligand, bonded to the Pt atom through the sulfur atom of Met 65, facilitates the entrance of the second one. This is an example of the kinetic trans effect, a well-known property of thioether ligands.

Bimetallic complexes are particularly fit for cross-linking of proteins. The metal–metal bonds or bridging ligands, or both, enrich the chemistry of these complexes and make them potentially versatile as reagents. Dirhodium (II) μ-tetracarboxylates, $[Rh_2(RCOO)_4]$, are suitable because the two unobstructed vacant coordination sites allow the formation of various adducts, $[Rh_2(RCOO)_4L_2]$.[30] The strong and short Rh(II)–Rh(II) bond and the entire lantern remain intact in the reactions with ligands L. The changeability of the group R permits purposeful adjustment of the hydrophilicity, lipophilicity, and Lewis acidity of the complex. The feasibility of the protein cross-linking through dirhodium complexes was demonstrated with $[Rh_2(OAc)_4]$ because it is the best-studied member of the series and because the strong ^1H NMR signal of the four equivalent methyl groups in the acetate bridges should permit a direct detection of the link despite the complexity of the protein ^1H NMR spectrum.[29]

A survey of the amino acids showed only cysteine, methionine, and histidine to react with $[Rh_2(OAc)_4]$. The diadducts with the last two amino acids and with imidazole were prepared as models for the possible diprotein complexes. The reaction with cysteine was not studied in detail because the horse cytochrome *c* lacks a free such residue. Incubation of the protein with $[Rh_2(OAc)_4]$ yielded $[Rh_2(OAc)_4(cyt)_2]$, which was purified by size-exclusion chromatography, as before; see Eq. (4).



(4)

The apparent molecular weight of the diprotein complex, determined by this chromtography, is 34 ± 1 kDa, or ca. 30% greater than the actual value. The mobility on the Sephadex column depends on the shape, as well as on the size, of a biological macromolecule. Since the $Rh_2(OAc)_4$ link keeps the protein ligands ca. 2.5Å farther apart then the $PtCl_2$ link does, the bimetallic diprotein complex deviated more from the idealized spheroidal shape than did the monometallic complex, and the error in its apparent molecular mass was somewhat larger.

Several pieces of evidence pointed at His 33 as the binding site. Whereas the ^1H NMR signals of the acetate bridges and of the many detectable amino-acid residues were virtually unperturbed, the signal of ^2C-H in the imidazole ring of His 33 was shifted downfield upon cross-linking. The difference UV-vis spectrum $[Rh_2(OAc)_4(cyt)_2]$ minus (2 cyt) nearly matched that of the model complex $[Rh_2(OAc)_4(Im)_2]$; small discrepancies were explicable in terms of the differences between the chromophore environments in the diprotein complex and in the model complex. The diprotein complex was formed at pH 7.0 but not at pH 5.0, because the pK value of His 33 is 6.4. Finally, the tuna cytochrome *c*, which has the unreactive tryptophane instead of histidine in position 33, failed to yield a diprotein complex with $[Rh_2(OAc)_4]$.

The complex $[Rh_2(OAc)_4(cyt)_2]$ is stable in solution but it was cleaved, and the native protein recovered, by incubation with 2-mercaptoethanol, a highly nucleophilic ligand. The diprotein complex proved more stable thermodynamically than would be expected on the basis of the equilibrium studies of $[Rh_2(OAc)_4L_2]$ model complexes with histidine and histidine-containing dipeptides as L.[31] Even the nucleophiles such as CN^- and N_3^-, present in excess, only partially displaced the protein molecules. The inertness of the diprotein complex may result from two causes. First, the protein molecules may shield the $Rh_2(OAc)_4$ core from attack by other potential ligands. Second, the oxygen atoms constituting the RhO_4 faces may form hydrogen bonds to the protein. Simulation by molecular graphics revealed a possibility of such a bond to the side chain of Asn 22.

The enhanced stability of the diprotein complex shows how metal complexes with small ligands, such as amino acids, may be unrealistic models for the complexes with biological macromolecules,

such as proteins. A greater structural complexity of the macromolecules and a variety of functional groups in them permit secondary interactions with the inorganic label or link that are impossible in amino-acid complexes.

The two types of reagents discussed above have already shown the benefits of an inorganic approach to the cross-linking of proteins. The $[PtL_2Cl_2]$ reagents are selective toward methionine,[28] whereas the $[Rh_2(RCOO)_4]$ reagents are selective toward histidine.[29] This useful contrast is a consequence of the well-known affinities of platinum and rhodium for the sulfur and nitrogen ligands, respectively. Those organic reagents that are selective—many are not—usually bind to amino or thiol groups, i.e., to lysine or cysteine residues. The different selectivity of the new inorganic reagents may prove advantageous in biochemical work.

Since the $[Rh(RCOO)_4]$ complexes and their homologs undergo one-electron oxidation reversibly and fast,[32] as in Eq. (5),

$$[Rh_2(RCOO)_4] \rightleftharpoons [Rh_2(RCOO)_4]^+ + e^- \qquad (5)$$

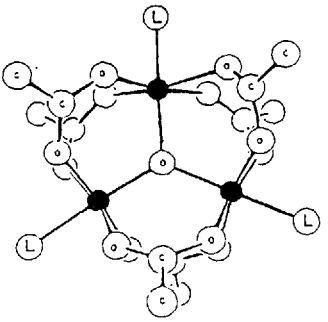
in principle they can be used as redox-active relays for electron-transfer reactions between the cross-linked metalloproteins. The complex $[Rh_2(OAc)_4]$ is ill-suited for this purpose because its oxidation potential, 1.40 V vs. NHE, is far above the reduction potential of ferricytochrome *c*, 0.26 V. Fortunately, the potential of the dirhodium complex is lowered markedly upon substitution of acetamidate, $CH_3C(O)NH^-$ (designated acam), for acetate in the bridging positions.[33] We have prepared the diprotein complex $[Rh_2(acam)_4(cyt)_2]$.[34] The oxidation potential of the bimetallic link in it is approximately 0.41 V, so that a mixed-valent Rh(II)–Rh(III) cation is accessible. Kinetic experiments concerning the electron transfer from the ferrocytochrome *c* to the mixed-valent link are going on.

FORMATION OF PROTEIN CLUSTERS[35]

Protein molecules evidently behave as ligands and form complexes—monosubstituted in the case of labeling, disubstituted in the case of cross-linking—with metal atoms. Perhaps their analogy

with conventional ligands can be extended to the formation of cluster complexes.

The trimetallic cluster $[Ru_3(\mu_3\text{-}O)(\mu_2\text{-}OAc)_6(H_2O)_3]^+$, one in a series of so-called basic metal carboxylates, is well-suited as a template for the formation of protein aggregates.[36] It is soluble in water; the H_2O molecules can be displaced by other ligands, L, in reactions that leave the rest of the cluster intact; and the triangular arrangement of metal atoms, shown below, leaves the greatest room for the bulky protein ligands as L.



$[Ru_3(\mu_3\text{-}O)(\mu_2\text{-}OAc)_6L_3]^{+/0}$

Incubation of horse cytochrome *c* with the aquo cluster at pH 7.0, followed by size-exclusion chromatography on Sephadex columns, yielded three fractions, whose relative molecular masses corresponded approximately to the protein trimer, dimer, and monomer. The characterization of the protein aggregates and their comparisons with model complexes containing methionine and histidine is in progress.

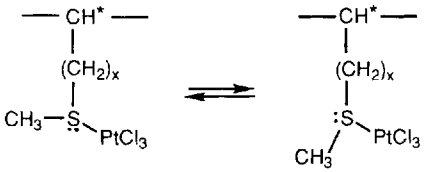
The reversible interconversion between the paramagnetic cationic cluster and the diamagnetic neutral cluster is shown in Eq. (6).

$$[Ru_3^{III}(\mu_3\text{-}O)(\mu_2\text{-}OAc)_6L_3]^+ + e^- \rightleftharpoons [Ru_2^{III}Ru^{II}(\mu_3\text{-}O)(\mu_2\text{-}OAc)_6L_3] \quad (6)$$

Since the potential for cytochrome *c* is above that for this couple (whose exact value depends on the ligand L), the mixed-valent cluster should be able to reduce ferricytochrome *c*. Our preliminary experiments with stopped-flow spectrophotometry indeed revealed a process that may be an electron-transfer reaction within the putative triprotein cluster.[35]

HYDROLYSIS OF PEPTIDE BONDS[37]

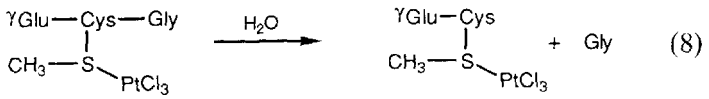
Our laboratory has introduced ^{195}Pt NMR spectroscopy as a method for the study of stereodynamic processes. So far, we have examined the inversion of configuration at the S atom in the Pt(II) complexes with methionine, cysteine, their derivatives, and organic thio ethers.[38,39] This inversion is shown in Eq. (7).



x = 0 - 3 (7)

Because the ultimate goal of this project is to probe the mobility of the protein surface with platinum tags, and because a ^{195}Pt NMR signal of a platinum-labeled biological macromolecule has not yet been observed, a gradual approach involving bioligands of intermediate size is necessary. Soon after such studies with peptides were started, however, an unexpected finding turned our interest from stereodynamics to analytical biochemistry.

The complex $[PtCl_3(GSMe)]^-$, in which S-methyl glutathione is coordinated to the Pt(II) atom as a unidentate thio-ether ligand, showed the expected two ^{195}Pt NMR signals of the two diastereoisomers. But another pair of signals grew in after some time, and eventually replaced the original pair. The ^{195}Pt NMR chemical shifts of the declining and growing pairs were very similar, an indication that the ligand set around the platinum atom remained unchanged. The ^1H NMR spectroscopy showed clearly the accumulation of free glycine in solution. Evidently, the Cys–Gly peptide bond was cleaved, as shown in Eq. (8).[37]



(8)

The unplatinated (free) tripeptide proved stable under the same conditions. Both the initial tripeptide complex and the final dipeptide complex exhibited diasteromerism owing to the chiral sulfur atom in the cysteine segment, but the slight difference in the environment (presence vs. absence of a terminal glycine residue) manifested itself in the ^{195}Pt NMR shifts. Despite some polymerization of $[PtCl_3(GS)]^{2-}$, in which glutathione is coordinated as a unidentate thiolate ligand, glycine was cleaved off of this complex, too. The cleavage reactions followed the first-order rate law, but their completion required several days regardless of the pH between 1 and 7. The rate depended on the anions present in solution—replacement of DCl with $DClO_4$ caused a significant acceleration of the hydrolysis.

The reaction is quite selective—the γGlu–Cys peptide bond was not cleaved. Control experiments with the $[PtCl_3L]^{n-}$ complexes containing N-acetyl-S-methylcysteine ($n = 1$) and N-acetylcysteine ($n = 2$) as L confirmed the relative inertness of the amide bond on the amino side of cysteine; the cleavage of this bond, and concomitant release of acetic acid, was very slow.

The mechanism of this selective hydrolysis can be theorized about. There are at least two possibilities. In the first one, the tethered Pt complex polarizes the carbonyl group, renders it more electrophilic, and enhances the attack by water. Because of the low ligating ability of the carbonyl oxygen atom, the concentration of the activated complex must be low and hence the slowness of the reaction. The selectivity is a result of stereochemical control. With the carboxyl group of cysteine the Pt tag can form a favorable six-membered ring, whereas with the γ-carboxyl group of glutamic acid it would have to form an unfavorable seven-membered ring. The peptides in $[Pt(dien)(GS)]^+$ and in $[Pt(trpy)(GS)]^+$, complexes containing the tridentate diethylenetriamine[40] or terpyridine[37] ligand instead of the unidentate chloride ligands, did not hydrolize. This fact indicates that the "productive" platinum–carbonyl interaction perhaps is a true cordination, involving a displacement of a chloride ligand. An alternative mechanism would involve hy-

drogen bonding of water to the coordinated chloride ligands and the delivery of such water molecules to the vicinity of the peptide bond to be cleaved.

In proteins, methionine as well as cysteine will react with $[PtCl_4]^{2-}$, but peptide bonds involving methionine should not be broken. An additional methylene group in the side chain of methionine would render both interactions—via the seven-membered and the eight-membered ring—stereochemically unfavorable. Preliminary experiments with hemoglobin and albumin, which contain free cysteine residues, showed protein cleavage after incubation with the $[PtCl_4]^{2-}$ complex.[37] The study of these and similar reactions should lead to the development of an inorganic, nonenzymatic method for the selective hydrolysis of peptides and proteins at the carboxyl side of the cysteine residues, a site not cleaved by the common proteolytic enzymes.

PROSPECTS

The studies reviewed herein point the ways for further research in several directions. (1) The $[Pt(trpy)Cl]^+$ complex can serve as a noninvasive probe of the protein topography and as the source of the stable $Pt(trpy)^{2+}$ spectroscopic tag. (2) Suitably located arginine residues in proteins may be involved in the binding of metal ions. The biochemical studies of such binding should be augmented with inorganic studies of metal–guanidyl complexes. (3) Cross-linking of metalloproteins via the redox-active dirhodium and triruthenium complexes may yield novel systems for the study of relayed electron transfer between proteins. (4) The simple $[PtCl_4]^{2-}$ complex and its derivatives hold promise as specific cleavers of peptides and proteins. Although such reactions would be stoichiometric rather than catalytic, their simplicity should make them routinely applicable.

CONCLUSIONS

Transition-metal compounds are well-suited for various applications in biochemistry and biophysics. Small complexes can label

or cross-link biomolecules, and clusters can aggregate them. The selectivity can be controlled on the basis of the well-known principles of coordination chemistry. The discrimination by an inorganic complex among the accessible potential ligands in a biomolecule depends on the identity, oxidation state, and hardness or softness of the metal; on the steric and electronic properties of the displaceable and ancillary ligands; and on the charge, size, and geometry of the complex as a whole. Judiciously chosen tags and links can be stable under ordinary conditions and yet easily removable. The spectroscopic and electrochemical properties of the metal complexes can permit their easy detection and quantitation on the biological macromolecules. The proteins can be labeled and cross-linked under mild conditions, without a large excess of the inorganic complex. Although the yield may not be high—some standard organic reagents give partial yields despite the great excesses used—the protein can easily be recovered and incubated anew. Repeated incubations can give a high overall yield while maintaining the advantages of the low concentration of the inorganic reagent, namely the selectivity of its binding and thus the homogeneity of the modified protein. When different modified derivatives or cross-linked aggregates are formed together, they can be separated chromatographically. Most important, the well-chosen inorganic reagents will bind to their targets noninvasively, so that the subsequent experiments with the modified biomolecules will be meaningful. Our studies with a few typical redox metalloproteins and proteolytic enzymes exemplify all of the aforementioned advantages of the inorganic reagents. But the widespread application of these reagents in bioinorganic, biochemical, and biophysical research is yet to come.

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three and a half years. The pertinent work from other laboratories is cited fully in the primary reports, Refs. 4, 5, 15, 21, 22, 27–29, 38, and 39. The drawing of $[Ru_3(\mu_3\text{-}O)(\mu_2\text{-}OAc)_6L_3]^{+/0}$ was adopted from D. N. Hendrickson *et al.*, Comments Inorg. Chem. **4**, 329 (1985) with permission of Gordon and Breach, Inc.

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