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Binuclear Transition-Metal Complexes as New Reagents for Selective Cross-Linking of Proteins. Coordination of Cytochrome *c* to Dirhodium(II) μ-Tetraacetate

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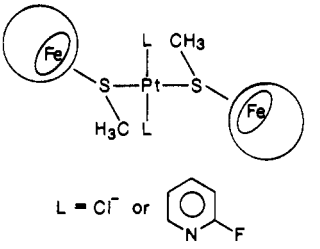
This study introduces binuclear transition-metal complexes as reagents for selective covalent cross-linking of proteins. Incubation of horse cytochrome *c* (designated cyt) with $Rh_2(OAc)_4$ under mild conditions yields the diprotein complex, $Rh_2(OAc)_4(cyt)_2$, whose composition is established by size-exclusion chromatography, UV-vis spectroscopy, and ^1H NMR spectroscopy. The protein molecules are coordinated to the Rh atoms via the imidazole (Im) rings of their His 33 residues, as shown by UV difference and ^1H NMR spectroscopy, by the pH effect on the complex formation, and by the control experiments with tuna cytochrome *c*. The diprotein complex is stable under ordinary conditions, and yet it can be cleaved, and the native protein recovered, by treatment with a suitable strong nucleophile. Spectroscopic and electrochemical measurements show that the structural and redox properties of cytochrome *c* are not perturbed significantly by cross-linking. Comparison between $Rh_2(OAc)_4(Im)_2$ and $Rh_2(OAc)_4(cyt)_2$ shows that the complex containing small ligands is not an entirely realistic model of the complex containing proteins. In particular, the enhanced stability of the latter toward hydrolysis may be due to steric bulk of the protein ligands and to hydrogen bonds that amino acid side chains may form with the inorganic link. Some of the findings of this study may pertain to the mechanism of antitumor action of the $Rh_2(RCOO)_4$ complexes.

I. Introduction

Many studies in biochemistry, biophysics, and chemical biology involved covalent cross-linking of enzymes and other proteins to different biomolecules and membranes.[1-5] All the reagents developed to date for this purpose are bifunctional organic compounds.[6] Some of them are selective, usually toward amino and sulfhydryl groups in the amino acid side chains; some, such as photogenerated nitrenes, are nonselective. Besides selectivity, desirable properties of cross-linking reagents are solubility in water; reactivity under mild, preferably physiological, conditions; stability; and cleavability, so that the linked species can be separated in their native forms.

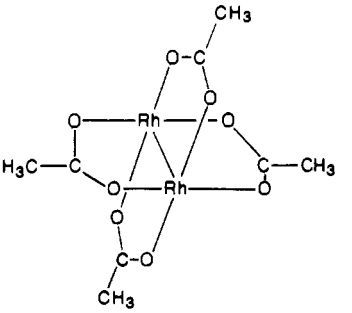
All of these required properties can be achieved with inorganic reagents. Various spectroscopic and chemical properties render transition-metal complexes uniquely suited for specific covalent binding to amino acid side chains in proteins and to other biological macromolecules.[7,8] Strong intraligand and charge-transfer absorption bands permit easy detection and quantitation and may depend on the side chain to which the complex is bonded; paramagnetic metal ions are useful as NMR relaxation agents and as EPR probes; heavy metals, with their high electron density, serve as labels for X-ray crystallography and electron microscopy. Chemical affinity of preformed complexes toward various competing binding sites can be controlled, and selectivity thus achieved, by the oxidation state of the metal and its hardness or softness, by the choice of ligands, and by the geometry and charge of the whole complex.

In a recent report from this laboratory[9] it was shown, for the first time, that proteins can be cross-linked selectively via transition-metal complexes. Incubation of horse-heart cytochrome *c* (designated cyt) with reagents $PtCl_4^{2-}$ and *trans*-$[Pt(2\text{-}Fpy)_2Cl_2]$ under mild conditions yields stable diprotein complexes *trans*-$[PtCl_2(cyt)_2]$ and *trans*-$[Pt(2\text{-}Fpy)_2(cyt)_2]$, respectively. (Symbol 2-Fpy stands for 2-fluoropyridine.) The thioether side chains of the Met 65 residues in the two proteins displace the two Cl^- ligands from the Pt(II) atom. Since the two ancillary ligands (Cl^- or 2-Fpy) remain, the properties of the PtL_2 link between the proteins are adjusted easily by the choice of the *trans*-$[PtL_2Cl_2]$ reagent. The structural and redox properties of the cytochrome *c* molecules



remain essentially unaltered upon cross-linking. Although the link is stable under ordinary conditions, it can be cleaved, and the native protein recovered, in a mild reaction.

This study introduces *bimetallic* complexes as reagents for cross-linking of proteins. The presence of metal-metal bonds or of bridging ligands, or both, enriches the chemistry of such complexes and makes them potentially versatile as protein links. Dirhodium(II) μ-tetracarboxylates, $Rh_2(RCOO)_4$, are especially suitable for binding to biological macromolecules for the following reasons.[10-13] Since each metal atom has an unobstructed vacant coordination site, various ligands readily form adducts of the types $Rh_2(RCOO)_4L$ and $Rh_2(RCOO)_4L_2$. The short and strong Rh(II)-Rh(II) bond and the entire $Rh_2(RCOO)_4$ lantern remain intact in the reaction with almost all of the ligands L. Variability of the bridging carboxylate, i.e., of the group R, permits purposeful changes in the hydrophilicity or lipophilicity of the complex and allows adjustment in the Lewis acidity of the metal atoms. The acetate complex, $Rh_2(OAc)_4$, is chosen because it is the most



widely studied member of the series and because the strong ^1H NMR signal of the four equivalent methyl groups should permit a direct detection of the complex even in the presence of the protein with its multitude of aliphatic C-H bonds.

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Previous bioinorganic study of the $Rh_2(RCOO)_4$ complexes was prompted by the discovery that they prolong significantly the survival times of tumorous mice.[14-19] In an attempt to elucidate the mechanism of the antitumor action and to develop useful drugs, Bear and co-workers have examined the effects of these complexes on several biosynthetic processes, and studied reactions between the complexes and various biological molecules.[20-27] Another possible oncological application was suggested in a recent study, which showed the $Rh_2(OAc)_4$ adducts with nitroimidazoles to be more efficient than the nitroimidazoles themselves as radiosensitizers of cells in vitro.[28] Although the main purpose of the present work is to develop $Rh_2(RCOO)_4$ complexes as selective reagents for the cross-linking of proteins, its finding are perhaps relevant to some of the questions raised in the study of these complexes as potential antitumor drugs.

II. Materials and Methods

Chemicals. The proteins, amino acids, amino acid derivatives, 4,4′-bipyridyl, and Sephadex G 75-50 were obtained from Sigma Chemical Co.; deuteriated solvents and $Rh_2(OAc)_4$, from Aldrich Chemical Co. Distilled water was demineralized further in a Barnstead Nanopure II apparatus.

Purification of Cytochrome c. Ferricytochrome c from horse heart (preparations of types III and VI from Sigma) was fully oxidized with $K_3[Fe(CN)_6]$; dialyzed in an Amicon ultrafiltration cell with a YM-5 membrane under the pressure of nitrogen, and chromatographed, with 85 mM phosphate buffer at pH 7.0 as an eluent, on a column of CM 52 cation exchanger obtained from Whatman.[29] Only the major fraction, containing ca. 85% of the commerical protein, was used in subsequent experiments.

Determination of Molecular Mass. Size-exclusion chromatography was performed on a column of Sephadex G 75-50, sized 1.5 × 70 cm, at 4 °C. The eluent was 85 mM phosphate buffer at pH 7.0. The flow rate of 10.0 mL h^{-1} was maintained with a peristaltic pump. Absorbance was measured with an ISCO V-4 detector equipped with a strip-chart recorder. The following proteins, whose molecular masses in kDa are given in parentheses, were used for calibration of the column before every determination: aprotinin (6.5), horse-heart cytochrome c (12.5), carbonic anhydrase (29.0), and bovine albumin (65.0). For greater accuracy, elution times at constant flow rate, rather than elution volumes, were measured.

Absorption Spectroscopy. The UV–vis spectra were recorded with an IBM 9430 spectrophotometer, whose monochromator contains two gratings. The spectra of the model complexes $Rh_2(OAc)_4L_2$ in aqueous solution were measured in the presence of excess ligand L to ensure complete formation of the diadducts.[30-34] Such precaution was not necessary with solutions in DMF. The IR spectra were recorded with an IBM IR98 Fourier-transform instrument, whose sample chamber was flushed with purified nitrogen. Dried $Rh_2(OAc)_4L_2$ complexes, lyophilized cytochrome c, and lyophilized $Rh_2(OAc)_4(cyt)_2$ complex were taken into Nujol mulls, which were then smeared onto the CsI plates.

Magnetic Resonance Spectroscopy. The ^1H NMR spectra at 300 MHz were recorded with a Nicolet NT300 spectrometer. The "inorganic" samples were dissolved in D_2O or in DMF-d_7, and the residual protons in these solvents were used as standards for chemical shifts. The protein-containing samples were dialyzed repeatedly into D_2O by ultrafiltration and then lyophilized with successive portions of D_2O in order to replace the exchangeable H atoms with deuterium.

The X-band EPR spectra were recorded at 5 K with a Bruker ER200-SRC instrument equipped with an Oxford Instruments ESR900 cryostat. A double rectangular cavity had a nominal frequency of 9.56 GHz; modulation frequency was 100 kHz. The protein-containing samples were fully oxidized with $[Co(phen)_3](ClO_4)_3$,[35] and the oxidant was removed by dialysis into 85 mM phosphate buffer at pH 7.0.

Voltammetry. Differential-pulse and cyclic voltammograms were obtained with an IBM EC225 electrochemical analyzer equipped with a Houston Instrument Omnigraph 200 XY recorder. A BAS cell assembly consisted of an Ag/AgCl couple as reference, a Pt wire as auxiliary, and a 1.6-mm Au disk as working electrode. The composition of the solution was as follows: ca. 0.5 mM in cytochrome c, 10 mM in 4,4′-bipyridiyl as a mediator, and 100 mM in $NaClO_4$,[36] all dissolved in 85 mM phosphate buffer at pH 7.0. A small, jacketed 5-mL cell permitted experiments with 2-mL samples. The solutions were deoxygenated by gentle bubbling of argon, and a blanket of this gas was maintained during the measurements.

III. Reactions with Amino Acids and with Cytochrome c

Survey of Amino Acids. Every amino acid containing a heteroatom in the side chain was incubated with $Rh_2(OAc)_4$ in the phosphate buffer at pH 7.0, at room temperature, for several days; the concentrations of the amino acid and of $Rh_2(OAc)_4$ were 10 and 1 mM, respectively. The following amino acids caused no change in the UV–vis spectrum under the general conditions used for the cross-linking of cytochrome c: Lys, Trp, Arg, Asp, Asn, Glu, Gln, Pro, Thr, Ser, and Tyr. Only with Cys, Met, and His were there changes in the spectra, and the color turned from bluish green to yellow, purple, and pink, respectively. In order to ensure that the coordination in these three cases occurs solely through the side chain, the experiments were repeated with N-acetyl derivatives of the amino acids, designated AcCys, AcMet, and AcHis, and with imidazole, designated Im. The changes in the spectra were identical with those observed with free amino acids.

$Rh_2(OAc)_4(Im)_2$. A suspension containing 100.0 mg (0.23 mmol) of $Rh_2(OAc)_4$ and 30.5 mg (0.46 mmol) of imidazole in 20 mL of acetone was stirred overnight. The color changed from bluish green to pink. The pink solid was removed by filtration, washed several times with acetone, and dried in vacuo at 56 °C overnight. Yield: 124 mg or 95%. Anal. Found (calcd): C, 28.86 (29.09); H, 3.64 (3.49); N, 9.46 (9.69).

$Rh_2(OAc)_4(MetH)_2$. This compound was prepared by a modified published procedure.[37] A suspension containing 44.2 mg (0.10 mmol) of $Rh_2(OAc)_4$ and 29.8 mg (0.20 mmol) of D,L-methionine in 20 mL of deoxygenated water was stirred, under nitrogen, for 3 h. The color changed from bluish green to purple, and the solution became clear. After evaporation to ca. 1 mL, the purple product was precipitated with excess acetone, filtered out, and washed several times with acetone. Yield: 56.4 mg or 76%.

$Rh_2(OAc)_4(cyt)_2$. Ferricytochrome c was incubated with $Rh_2(OAc)_4$ in 85 mM phosphate buffer at pH 7.0. The protein concentration was 2.0 mM, usually 25 mg (2.0 μmol) in 1.0 mL of the buffer. The concentration of the linking reagent was varied systematically from 1.0 to 5.0 mM, i.e., from 0.44 to 2.2 mg (1.0 to 5.0 μmol) in 1.0 mL of the buffer; incubation time was also varied. The optimal yield, 20% on the average, was achieved with

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2.0 mM protein and 5.0 mM $Rh_2(OAc)_4$ in 2 days.

Size-exclusion chromatography of the reaction mixture yielded three well-separated major bands. The first two of them, designated bands I and II, contained cytochrome *c* and were red-brown. Their apparent molecular mass (averages of several measurements) were 34 ± 1 and 12.5 ± 0.5 kDa, respectively. These values correspond respectively to two molecules and one molecule of cytochrome *c*. (The discrepancy between the measured and expected molecular masses of the diprotein complex is explained in section IV.) The third band, designated band III, contained the unspent $Rh_2(OAc)_4$ and was bluish green. When the incubation was carried out at pH 5.0 (in 85 mM acetate buffer), no cross-linking took place. Only a red-brown band of the unspent cytochrome *c*, having the molecular mass of 12.5 kDa, and a blue band of the unspent $Rh_2(OAc)_4$ were obtained.

The cross-linking experiments (incubation at pH 7.0 and size-exclusion chromatography) were performed in the standard way with horse-heart cytochrome *c* tagged at His 33 with a Pt(trpy)$^{2+}$ group[38] and with the native tuna-heart cytochrome *c*. In the former case the yield of the diprotein complex was much lower (ca. 5%) than with the native horse-heart cytochrome *c*. In the latter case the diprotein complex was not obtained at all. The significance of these findings is discussed in section V.

Stability of the Diprotein Complex. Complex $Rh_2(OAc)_4(cyt)_2$ was incubated at room temperature, in 85 mM buffers at different pH values, without or with added nucleophiles, and the composition of the solutions was determined after 2–3 days by size-exclusion chromatography. All the solutions were 50 μM in the diprotein complex and 100 μM, 1 mM, or 100 mM in the nucleophile. The cleavage yield is the ratio between the amounts of the monomeric and of the total (monomeric plus dimeric) cytochrome *c*. Incubation at pH 7.0 (in phosphate buffer) produced less than 5% of the monomeric cytochrome *c*; similar incubation at pH 5.0 (in acetate buffer) produced 16% of it; the presence of a 2-fold excess of NaN_3 at pH 7.0 caused the cleavage yield of 8%; both 2-fold and 20-fold excesses of NaCN at pH 7.0 raised this yield to 30–35%; 2000-fold excess of 2-mercaptoethanol at pH 7.0 caused nearly complete (greater than 80%) displacement of the protein molecules from the $Rh_2(OAc)_4(cyt)_2$ complex.

IV. Coordination of Amino Acids and Cytochrome *c* to $Rh_2(OAc)_4$

Reactions with Amino Acids. Early in the study of axial coordination to $Rh_2(RCOO)_4$ it was noted that the color of the adducts depends markedly on the ligand, especially on the identity of the donor atom.[39-42] Four characteristic absorption bands, two of them in the visible and two in the UV region, have been identified and assigned.[43-46] The band at the lowest energy is particularly sensitive to the identity and properties of the donor atom in the axial ligand. This sensitivity was exploited in our survey of the coordinating ability of the various amino acids and their derivatives toward $Rh_2(OAc)_4$. Since most of the amino acids did not affect the UV–vis spectrum of this complex, the α-amino group must be unreactive. The carboxylate group would be a weak ligand, unlikely to form a stable adduct in aqueous solution.[10-13] Of the side chains, only the sulfhydryl group (of cysteine), imidazole (of histidine), and the thioether (of methionine) proved reactive toward $Rh_2(OAc)_4$. Since the reactions with cysteine and other thiolates have been studied before[23,47,48] and since cytochromes *c* used in the cross-linking experiments do not contain a free cysteine residue, the adducts with this amino acid were not examined further. This study focused on the reactions with histidine and methionine, amino acids that are shown below:

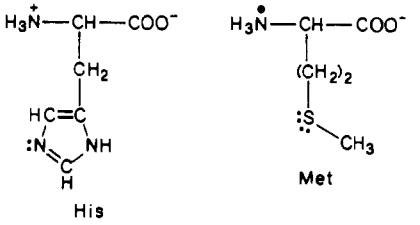

Table I. Spectroscopic Properties of Model Complexes with Imidazole and Methionine

property	$Rh_2(OAc)_4$[a]	$Rh_2(OAc)_4(Im)_2$	$Rh_2(OAc)_4(Met)_2$
UV–vis abs max, nm (ϵ, M^{-1} cm^{-1})			
85 mM phosphate, pH 7.0[b]	219 (13 800)	268 (12 700)	308 (34 700)
	245 sh		
	446 (100)	434 sh	445 sh
	588 (210)	517 (210)	537 (330)
DMF[c]	264 (8400)	267 (9200)	307 (27 100)
	445 (120)	451 (120)	
	587 (270)	550 (240)	534 (330)
far-IR bands, cm^{-1} [d]	631 m	630 s	625 s
	621 m	595 m	591 m
	602 m	381 vs	381 vs
	395 s	339 s	328 s
	381 s		
	354 vs		
^1H NMR, ppm vs TMS[e]	1.75 (1.88)[f]	1.71 (6)	
		7.53 (1)	
		7.63 (1)	
		8.35 (1)	

[a] Unoriginal measurements, made for the sake of reliable comparisons. [b] Concentrations: Im or Met, 200 mM; $Rh_2(OAc)_4$, 50 μM for UV measurements and 1 mM for vis. [c] Isolated model complexes. [d] In Nujol mulls. [e] In DMF-d_7; all signals are singlets; relative intensities in parentheses. [f] The value in parentheses is for the solution in D_2O.

Model Complexes. The spectroscopic properties of the $Rh_2(OAc)_4L_2$ adducts with imidazole and methionine as ligands L are given in Table I. Their UV–vis spectra agree with those reported for similar compounds.[49] The ^1H NMR signal of the μ-OAc ligands remains virtually unaffected by adduct formation, but the signals of the axial ligands are affected in the usual way; the magnitude of the downfield shift caused by coordination decreases as the number of bonds between the H atom in question and the donor atom increases. The IR stretching frequencies of the $Rh_2(OAc)_4$ unit are not changed significantly by adduct formation. The structure of the bimetallic lantern evidently is maintained in the adducts.

$Rh_2(OAc)_4$ was treated with the two possible ligands at pH values of 5.0 and 7.0. Whereas methionine gave the diadduct under both conditions, histidine failed to do so at pH 5.0. The pK_a value of the imidazole side chain is 6.0,[50] and the imidazolium form evidently is less reactive than its conjugate base.

Cross-Linking of the Protein. The formation of the diprotein complex is shown in eq 1. Although its yield—20% on the average—is not high, it is achieved under mild conditions, without use of a large excess of the bimetallic complex. Many standard organic reagents do not give much higher yields even when applied in a large excess. The monomeric cytochrome *c* is easily recovered by size-exclusion chromatography and may be incubated anew. Repeated incubations give a high overall yield while maintaining the advantages of the low concentration of $Rh_2(OAc)_4$, namely the selectivity of its binding to the protein and the homogeneity

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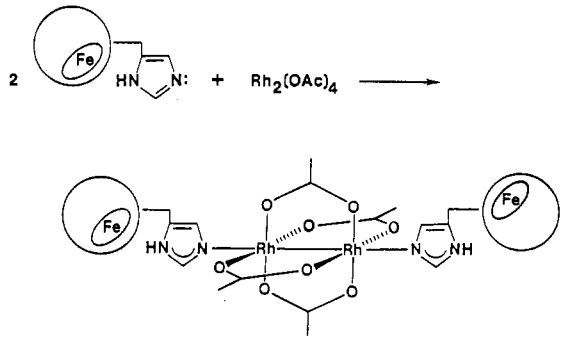

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of the resulting diprotein complex.

The molecular mass of 34 ± 1 kDa is greater than the value expected for $Rh_2(OAc)_4(cyt)_2$. The relative error of about 30% becomes understandable in view of the mechanism of size-exclusion (gel-filtration) chromatography. A strict proportionality between the elution time and the log M_r—molecular mass itself is only an approximate measure of the biopolymer size—obtains only with protein molecules of similar shapes. (The same is true about SDS gel electrophoresis.) The globular, single-chain proteins that are used as calibration standards indeed define a linear plot of log M_r versus elution time. Linking of two spheroidal molecules of cytochrome *c* via a bimetallic bridge, however, results in an elongated diprotein complex, which, to the Sephadex gel, appears larger than a spheroidal protein of the same molecular mass. This explanation is borne out by the comparison between the present diprotein complex and *trans*-$[PtCl_2(cyt)_2]$, whose molecular mass of 30.7 ± 0.8 kDa deviates from the expected value by 18%.[9] Since the $Rh_2(OAc)_4$ link keeps the protein ligands ca. 2.5 Å farther apart than the $PtCl_2$ link does, the bimetallic diprotein complex deviates more from the idealized spheroidal shape than does the monometallic complex, and the error in its apparent molecular mass is somewhat larger. The UV difference spectrum (discussed in section V) proves the cyt:$Rh_2(OAc)_4$ ratio to be 2:1.

The diprotein complex $Rh_2(OAc)_4(cyt)_2$ is more stable thermodynamically than would be expected on the basis of the equilibrium studies of $Rh_2(OAc)_4L_2$ model complex with histidine and histidine-containing dipeptides as ligands L.[30,31,34] Prolonged standing at pH 7.0, at room temperature, does not cause appreciable dissociation of the diprotein complex. Even the good nucleophiles such as CN^- and N_3^- anions, present in excess, only partially displace the protein ligands. A large excess of the highly nucleophilic 2-mercaptoethanol, however, can extrude the linking reagent and restore the native cytochrome *c*.

The enhanced stability of the protein complex may result from two causes. First, the bulky and rather hydrophobic protein molecules may shield the bimetallic link from attack by the solvent (water) and by other potential ligands present in solution. Second, the oxygen atoms constituting the RhO_4 faces may form hydrogen bonds with side chains of some amino acid residues. Simulation by molecular graphics of $Rh_2(OAc)_4$ binding to the imidazole ring of His 33 corroborates this second hypothesis. At the Rh–N distance of 2.23 Å, typical for this bond,[50-55] one of the O atoms approaches the amide N atom in the side chain of Asn 22 at 2.32 Å. Such hydrogen bonding has indeed been found in $Rh_2(OAc)_4$ adducts with thiamin,[56,57] theophylline,[58] and caffeine[58] and has been proposed for the adduct with adenine.[58] A more evidential consideration of hydrogen bonding would require a crystallographic analysis of the diprotein complex.

The enhanced stability of the diprotein complex is an example of how metal complexes with small ligands, such as amino acids, may not be entirely realistic models of the complexes with biological macromolecules, such as proteins. The chief differences between the ligands of the two types, besides shear size, are the possession by the macromolecules of the secondary and tertiary structures and the greater variety of functional groups that they contain.

Table II. Electronic and Structural Characteristics of the Native and Cross-Linked Horse-Heart Cytochrome *c*

property	cyt	$Rh_2(OAc)_4(cyt)_2$
UV–vis abs max, nm[a]	279.6	278.6
	360.8	360.0
	410.0	408.2
	530.8	530.6
	693.6	691.6
difference UV max, nm (ϵ, M^{-1} cm^{-1})[a,b]		275 (13 500)
reduction pot., mV vs NHE[c]	262 ± 5	252 ± 5
EPR *g* values[a]		
g_x	1.24	1.30
g_y[d]	2.31	2.36
g_z	3.14	3.07
$g_\parallel$	2.03	2.03
$g_\perp$	6.05	6.02
^1H NMR resonances, ppm[e]		
CH_3 in ring IV	35.39	35.27
CH_3 in ring II	32.32	32.24
CH_3 in Met 80	−24.4	−24.9
S-CH-CH_3 in ring II	−2.44	−2.40
S-CH-CH_3 in ring I	−2.74	−2.73
C(2)-H in His 33	7.91	8.43
aromatic CH	6.84	6.82
CH_2 in Tyr and Phe	2.98	2.92
β-CH_2 in 3 Glu and CH_3 in Met 65	2.09	2.09
β-CH_2 in 9 Glu	1.95	1.94
β-CH_2 and γ-CH_2 in Lys	1.63	1.63
CH_3 in Ala and γ-CH_2 in Lys	1.47	1.44
CH_2 in Ile	1.33	1.30
CH_3 in Val	0.95	0.95
CH_3 in Ile	0.84	0.84
CH_3 in Leu	0.89	0.90
CH_3 in μ-OAc		1.89

[a] In 85 mM phosphate buffer at pH 7.0. [b] $Rh_2(OAc)_4(cyt)_2$ − 2 cyt. [c] In 85 mM phosphate buffer at pH 7.0 and 25 °C. [d] At the peak maximum. [e] Downfield from DSS, with respect to the residual water as standard.

Structural and Redox Properties of the Cross-Linked Protein Molecules. The complex $Rh_2(OAc)_4(cyt)_2$ was compared with the native cytochrome *c* by various physical methods in order to determine whether the linkage alters the protein structure and properties of its active site. The findings are given in Table II.

The EPR spectra confirm that the electronic structure of the ferriheme is virtually unperturbed; the slight differences between the *g* values fall within the error limits of the measurement.[59-61] A small amount (less than ca. 10%) of the protein exists in the high-spin ferric form, characterized by *g* values of about 2.0 and 6.0. Since the UV–vis absorption maxima are not altered significantly, whereas the Soret and other bands are known to move to much shorter wavelengths in high-spin heme proteins,[62] this form must be a very minor constituent of the protein dimer.

Particularly diagnostic is the absorption band at ca. 693 nm, whose intensity depends on the interactions between the Fe atom and its axial ligands, Met 80 and His 18.[63-65] Although the absorptivity is too low to permit accurate comparisons of the band intensity between the proteins, the presence of this band in the

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spectrum of the dimer indicates that the Fe atom remains hexacoordinate. Dissociation of the Met 80 ligand, a process detectable even in the native cytochrome *c* and responsible for conversion to the high-spin form,[66] may be enhanced slightly upon cross-linking.

The reduction potential of the diprotein complex is virtually identical with that of the native protein. This property depends markedly on the spin state of the heme; for example, the value for the high-spin cytochrome *c* from *Rhodopseudomonas rubrum* is 10 mV at pH 7.0.[67] The high-spin protein must be a very minor fraction of $Rh_2(OAc)_4(cyt)_2$.

The proton NMR spectrum of the active site in heme proteins depends markedly on the interactions between the paramagnetic Fe(III) atom and its axial ligands and between the heme periphery and the side chains of neighboring amino acid residues.[68-73] Particularly sensitive to the protein conformation are the hyperfine ^1H shifts,[74-78] which are evident in the first five resonances listed in Table II. Also informative are the data regarding many side chains.[75] Although the resonances in the diprotein complex are broader than their counterparts in the native cytochrome *c*, an expected consequence of the reduced mobility and of the lower symmetry, the ^1H NMR chemical shifts evidently are not altered significantly upon the cross-linking.

The CH_3 groups in the bridging acetato ligands proved particularly advantageous for the straightforward detection and quantitation of the link in the diprotein complex. Their ^1H signal is virtually unperturbed by the cytochrome *c* coordination because this H atom is four bonds removed from the rhodium atom. The signal is clearly evident in the NMR spectra of $Rh_2(OAc)_4(cyt)_2$ and of the mixture containing cytochrome *c* and $Rh_2(OAc)_4$ in the molar ratio 2:1 but is absent from the spectrum of the native protein. The relative intensities of the OAc and protein signals in the diprotein complex are approximately equal to the relative intensities of these signals in the mixture. This control experiment confirmed the composition of the diprotein complex to be $Rh_2(OAc)_4(cyt)_2$, the fact established independently by the measurement of the molecular mass.

V. The Binding Site

The Candidates. Only cysteine, histidine, and methionine form adducts with $Rh_2(OAc)_4$ under the conditions used for the protein cross-linking. Horse-heart cytochrome *c* does not contain free cysteine but contains histidine in positions 18, 26, and 33 and methionine in positions 65 and 80. Binding to His 18 and Met 80, axial ligands to the heme, can be ruled out. The ferriheme absorption bands at 410 and 530 nm and the one at ca. 693 nm, which is diagnostic of the interactions between the Fe atom and its axial ligands, are not significantly perturbed by cross-linking. The reduction potential, EPR *g* values, and hyperfine ^1H NMR shifts all essentially retain their native values. The possible binding sites, therefore, are Met 65, His 26, and His 33. The actual one is identified by several methods.

Effects of pH. Cytochrome *c* from horse can be cross-linked with $Rh_2(OAc)_4$ at pH 7.0, but not at pH 5.0, although the protein maintains the same conformation at these two pH values. This contrast indicates that the binding site in cytochrome *c* is an amino acid whose side chain undergoes protonation and deprotonation with the pK_a between these two values. Methionine, whose side chain is a thioether, clearly does not meet the requirement. Only one of the two His residues does; His 33 has the normal pK_a value of 6.4, whereas His 26 has the anomalous value of 3.2.[79] Both of these residues lie on the protein surface but in different environments.[80-83] The former belongs to a hydrophilic region and is exposed to the exterior, whereas the latter is hydrogen-bonded in a hydrophobic pocket. Only His 33 is easily accessible from solution, a fact established by our previous studies of the labeling of cytochromes *c* with $[Pt(trpy)Cl]^+$.[38]

Although $Rh_2(OAc)_4(cyt)_2$ does not form at pH 5.0, once formed it is relatively stable at that pH value. This contrast shows once again the enhanced stability of the adduct containing protein ligands. Effect of pH on coordination to $Rh_2(OAc)_4$ has been observed with adenosine monophosphate (AMP)[84] and with thiamin monophosphate (vitamin B_7).[57]

Absorption and ^1H NMR Spectra. As Table I shows, the model complexes with imidazole and methionine exhibit distinct UV–vis spectra. The spectrum of the inorganic chromophore between the protein molecules is obtained by subtracting the spectrum of the native cytochrome *c* from that of the diprotein complex: $Rh_2(OAc)_4(cyt)_2$ minus 2 cyt. The weak bands in the visible region are obscured by the stronger protein absorption, but the UV band is prominent. The comparison of the difference spectrum (the entry in Table II) with the spectra of the model complexes (the entries in Table I) rules out methionine and corroborates histidine as the axial ligand. The difference spectrum nearly matches that of $Rh_2(OAc)_4(Im)_2$ but is completely different from that of $Rh_2(OAc)_4(Met)_2$. The 6-nm shift of the band maximum and the slight change in its absorptivity (intensity) in the difference spectrum are understandable in view of the partial charge-transfer character of the corresponding electronic transition.[43-46] Such transitions are sensitive not only to the immediate ligands, which are imidazole rings both in the diprotein complex and in the model complex, but also to the more distant environment of the chromophore, which is the protein in the former and the solvent in the latter case. The absorptivity of the inorganic chromophore (ϵ_{275} = 13 500 M^{-1} cm^{-1}), calculated on the basis of the known absorptivities of cytochrome *c*, is an additional proof that the ratio cyt:$Rh_2(OAc)_4$ is 2:1.

The ^1H NMR spectrum of $Rh_2(OAc)_4(cyt)_2$ contains a signal at 2.09 ppm, exactly the position for the unperturbed CH_3 group in Met 65. The signal at 7.91 ppm, characteristic of the imidazole C(2)–H group, is absent, but a new one at 8.43 ppm is present. This new signal may be due to the coordinate imidazole (see Table I). This evidence confirms that the protein binds to the Rh atom via His 33 and not via Met 65.

Control Reactions. The assignment of the binding site is confirmed by the cross-linking experiments involving a labeled horse cytochrome *c* and the native tuna cytochrome *c*. Tagging of His 33 in the horse protein with $Pt(trpy)^{2+}$, a procedure reported in detail in our previous publication,[38] caused a great reduction of the cross-linking yield. A little (ca. 5%) of the $Rh_2(OAc)_4(cyt)_2$ complex still formed probably because the dirhodium complex partially displaced the platinum complex from His 33.

The protein from tuna is homologous to its congener from horse and contains all the potential binding residues except one—in position 33 it has tryptophan, an amino acid proven unreactive toward $Rh_2(OAc)_4$. Since the tuna protein yielded no Rh_2-

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$(OAc)_4(cyt)_2$ complex, it can be concluded that the site of cross-linking in the horse protein is His 33.

Selectivity. This and a previous study[9] from this laboratory have already shown the potential versatility of transition-metal complexes as cross-linking reagents for proteins. Whereas the complexes $PtCl_4^{2-}$ and *trans*-$[Pt(2\text{-}Fpy)_2Cl_2]$ are specific toward methionine side chains (in cytochrome *c* it is Met 65), the complex $Rh_2(OAc)_4$ is specific toward histidine side chains, provided the imidazole ring is accessible from solution. This selectivity of the bimetallic reagent, observed herein with cytochromes *c*, is likely to be found in experiments with other proteins that lack exposed cysteine residues. When such residues are present—the number of proteins containing them is relatively small—they will probably be the binding sites.

The selectivity of both the platinum and dirhodium reagents is consistent with the known affinity of these metals toward ligands. In particular, aromatic amines, imines, and other molecules with trigonal nitrogen atoms are the most common axial ligands in $Rh_2(RCOO)_4L_2$ complexes, and structures of many such adducts have been determined crystallographically.[50-55]

VI. This Study in the Context of Others

Discovery of the antitumor activity of $Rh_2(RCOO)_4$ complexes[14-19,26] spurred the research into their interactions with biomolecules. Although some of these studies involved macromolecules (nucleic acids and proteins),[20,21,23,24] most of them dealt with complexes of the corresponding small molecules (nitrogenous bases and amino acids).[22,27,47,48,85] In other studies, the dirhodium complexes were chosen as typical transition-metal compounds and their interactions with a vitamin[57] and with a model for a DNA-binding drug[86] were examined. Although the investigations of model complexes proved informative and useful in various ways, a concern was voiced about their relevance to the study of complexes with large biomolecules.[31] The enhanced hydrolytic stability of $Rh_2(OAc)_4(cyt)_2$ over that of the model complexes may be an example of such incomplete analogy between amino acids and proteins as ligands for transition metals. Both the relevance[24,27] and the irrelevance[25] of axial ligation to the antitumor effects of $Rh_2(RCOO)_4$ have been argued. The importance of size and of structural complexity of the biopolymers for their interactions with $Rh_2(RCOO)_4$ should be considered in the study of this question.

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Effects of Anions on the Removal of Iron from Transferrin by Phosphonic Acids and Pyrophosphate

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It has been reported previously that phosphonate ligands can remove the ferric ion from human serum transferrin by parallel reaction pathways, one which shows saturation behavior and one which is first-order with respect to the competing ligand (Harris, W. R.; et al. *Inorg. Chem.* **1987**, *26*, 2711–2716). In the present work the rates of iron removal from diferric transferrin by the chelating agents pyrophosphate (PP_i), nitrilotris(methylenephosphonic acid) (NTP), and *N*,*N*-bis(phosphonomethyl)glycine (DPG) have been measured in 0.1 M, pH 7.4 hepes buffer containing 200 mM concentrations of the sodium salt of the following anions: fluoride, chloride, nitrate, thiocyanate, bicarbonate, sulfate, and perchlorate. All these anions increase the rate constant for the first-order pathway for iron removal. The effects of anions on iron removal do not correlate with the lyotropic number of the anion or the iron-binding affinity of the anion. Instead, there appears to be a very specific effect of perchlorate on the first-order process. This observation supports the hypothesis of a selective anion-binding site near the C-terminal binding site of transferrin. The anions have varying effects on the saturation pathway for iron removal. The anions tend to increase the maximum rate constant for the saturation pathway for PP_i. However, the anions virtually eliminate the saturation pathway for NTP and tend to reduce the maximum rate of iron removal from this pathway by DPG. It is proposed that the high salt concentrations interfere with the binding of the phosphonic acids as anions to cationic amino acid residues near the iron-binding site. Initial rates of iron removal by PP_i and NTP have been measured as a function of the chloride and perchlorate concentrations. Both anions produce a linear increase in the initial rates of iron removal from diferric transferrin. Rates of iron removal by PP_i and NTP from both forms of monoferric transferrin have also been measured. Both chloride and perchlorate accelerate the rate of iron removal from C-terminal monoferric transferrin but reduce the rate of iron removal from N-terminal monoferric transferrin.

Introduction

Transferrin is the mammalian protein that carries ferric ion through the blood between sites of uptake, utilization, and storage.[1-3] The protein consists of two major lobes, each containing a single high-affinity iron-binding site. Although these sites are very similar, they are not identical. A distinctive feature of transferrin is the requirement of a synergistic anion for effective metal binding. Under physiological conditions the anion is (bi)carbonate, which appears to bind simultaneously to the ferric ion and to cationic groups on the protein to form an Fe–HCO_3-Tf ternary complex.

The iron binding constants of Tf are $10^{20.7}$ and $10^{19.4}$ at atmospheric CO_2 and pH 7.4.[4] It is not clear how cells overcome this high binding affinity to procure their iron. Exchange of iron at pH 7.4 with low-molecular-weight ligands tends to be quite slow,[5-7] and the mechanism by which this exchange takes place is still not completely clear. A better understanding of iron removal from transferrin takes on added importance because of the need

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