Reactions of Complexes of Platinum Metals with Bio-Molecules

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There is a growing recognition of the opportunities that may exist for inorganic biochemistry to contribute to the solution of practical problems that occur in many quite different areas, ranging from mineral extraction to medicine. While most traditional applications of the platinum metals depend upon their ability to remain largely unchanged in highly reactive environments, compounds of these metals can be quite reactive and do react with biological materials. This paper reviews the present position and indicates potential applications.

The reactions of co-ordination complexes of the platinum metals and gold with bio-molecules are relevant to a number of areas. The most important of these currently relates to the medicinal applications of platinum and gold (1), with much potential still to be explored. There are other applications, however, including the laboratory use of platinum metals as heavy-atom markers in the determination of biological macromolecule crystal structures, the use of osmium tetroxide as a specific oxidant and biological stain, and the use of attached ruthenium complexes as spectroscopic labels and probes for studying the electron-transfer reactions of bio-molecules. At the other extreme there is the potential use of micro-organisms in mining processes, and for the selective extraction/recovery of metals from aqueous solutions (pH>2) with cells or cell extracts. The formation of ruthenium as a fusion product in nuclear reactors—the isotopes [103]Ru and [106]Ru have half lives of 40 days and 1 year, respectively—and its release into the atmosphere at the time of the Chernobyl mishap, is also relevant.

The use of the Pt(II) complex *cis*-platin, *cis*-[Pt(NH$_3$)$_2$Cl$_2$], in chemotherapy was approved as recently as 1979, and this is now the leading anti-cancer drug in the U.S.A. (2). It is used in combination therapy with either adriamycin or bleomycin and binblastine, and finds applications in the treatment of testicular, ovarian and lung cancers. *Cis*-platin was the first successful anti-cancer drug, but it does display unpleasant side effects, notably kidney toxicity, nausea and vomiting, as well as neurotoxicity. Second generation drugs, carboplatin [a], which is now approved for use in the U.K., iproplatin [b] and spiroplatin [c], are less toxic and have better anti-tumour activity. (3). There are additional advantages, thus spiroplatin has higher water solubility, of 0.5M, which allows easier administration of the drug. It has been demonstrated that this form of drug is active against three animal tumours with a 30-fold greater activity than *cis*-platin. The use of carboplatin and iproplatin in synergistic combination with radiotherapy is being investigated. Third generation drugs which contain combinations of malonate and cyclobutanedicarboxylate with amine and diamine ligands, so giving a broad spectrum of activity, are currently under investigation (3, 4). Features of all these drugs are the use of neutral Pt(II) or Pt(IV) complexes which contain two cis amine groups (primary or secondary, but not tertiary) and two other good cis leaving groups. Thus trans complexes, or cis complexes with poor leaving groups, that is the inert CN$^-$, ONO$^-$, NCS$^-$ or I$^-$, are inactive.

Early experiments demonstrated that *cis*- and *trans*-platin bind more strongly to RNA than to

[a] Carboplatin   [b] Iproplatin   [c] Spiroplatin
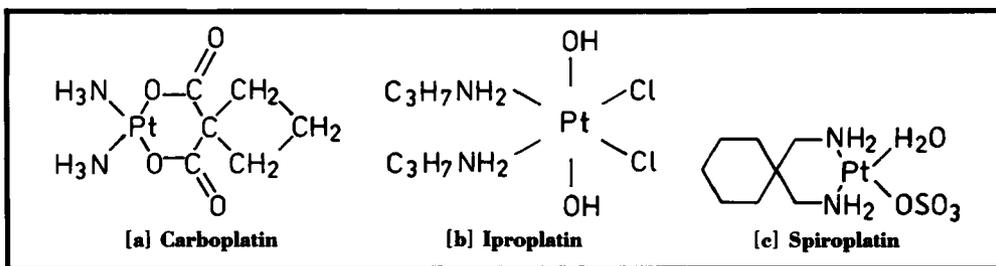
DNA, and least strongly to proteins. However, when their ability to suppress synthesis was measured at low concentrations only the synthesis of DNA was suppressed. It is believed that *cis*-platin type complexes are effective because they bind to the N-7 positions of two adjacent guanines of the DNA, and that this hinders replication (5–7). A normal cell is able to oppose this attachment by means of its repair mechanisms, while a tumour cell has a deficiency in repair proteins which could otherwise recognise the damaged segment and cause a repair. Analogous trans complexes attach more readily to guanine in the initial stages but decrease in concentration over 24 hour periods due to their removal by repair proteins.

A complex such as *cis*-platin is believed to remain in the neutral dichloro form in the plasma where the concentration of Cl⁻ is high (~ 103mM). After diffusion across the cell membrane, in the presence of only 4mM Cl⁻ in the cytoplasm, the complex yields the various aquated products (8). Since H_2O is a good leaving group the platinum can then attach itself to the DNA.

Rhodium complexes, for example $[Rh_2(O_2CR)_4]$, have been reported to possess some activity as anti-cancer agents (9). More generally, when added to cell growth media, complexes *trans*-$[RhX_2L_4]$ (where L is a N-heterocyclic such as pyridine) are known to interfere with cell division and cause filamentation in growth (10). A series of Pd(II) analogue complexes have been tested, but show little promise. It is known that Pd(II) is more labile than Pt(II) and this can lead to unavoidable toxicity. The Pd(II) may not even reach the DNA. Recent reports indicate that the four-coordinate

Au(I) bis-diphosphine complexes such as $[Au(dppe)_2]Cl$ may have a future as anti-cancer agents (11). Titanocene dichloride, $[Ti(Cp)_2Cl_2]$, where Cp = cyclopentadienyl, represents an interesting example of an organometallic anti-tumour reagent (12).

Another type of interaction of simple transition-metal complexes with DNA is the intercalation which occurs with planar heterocyclic chromophores. The heterocyclic ligands can insert and stack between base pairs of the DNA helix. Such effects have recently been reviewed by Barton (8). The stereoselective interaction of tris(phenanthroline) complexes such as $[Ru(phen)_3]^{2+}$ has for example been noted, the Δ-$[Ru(phen)_3]^{2+}$ complex intercalating more favourably than the Λ isomer, which is inhibited by steric repulsions between H-atoms of the phenanthroline and O-atoms of the DNA phosphate (13). There are also spectroscopic applications, and the tris (4,7 - diphenylphenanthroline) ruthenium (II) complex can be used as a sensitive stain for helix conformation in chromosome studies using fluorescence techniques. Electron-transfer reactions of intercalated metal complexes, for example the Fe(II)-EDTA derivative complex which has the aromatic methidium intercalator attached by a short hydrocarbon chain, can with oxygen give rise to single strand DNA scissions (14). The Fe(II) activates the oxygen to yield O_2^- or OH˙ radicals (or Fe-O₂ complexes), which at high local concentrations can bring about cleavage of the sugar phosphate backbone.

The binding of platinum complexes to cytochrome c has recently been studied. A *metallo*protein has many advantages in such

investigations because structural features have been extensively explored, and the presence of the metal helps in demonstrating whether attachment of a second metal has any disruptive effects on the protein structure. Kostič has shown that $[PtCl_4]^{2-}$ and $[Pt(2\text{-}Fpy)_2Cl_2]$, where 2-Fpy is 2-fluoropyridine, cross-link horse cytochrome c by co-ordinating to the S-atom of the thioether side chain of a methionine, [d], in this case Met65 (15). The binding is illustrated in [e]. With $[Pt(terpy)Cl]^+$ however, selective covalent labelling of histidine residues, [f], His33 (major binding) and His26 (minor binding) is observed, with no labelling of Met65 (16). The difference in behaviour can be attributed to the steric demands of the terpyridine ligand, which prevent it binding to the thioether group. Interestingly, in the case of the complex $[Pt(NH_3)_3H_2O]^+$ preliminary results indicate that binding occurs 55 per cent to the methionine and 45 per cent to histidine His33 (17).

Met
[d]

[e]

His   [f]
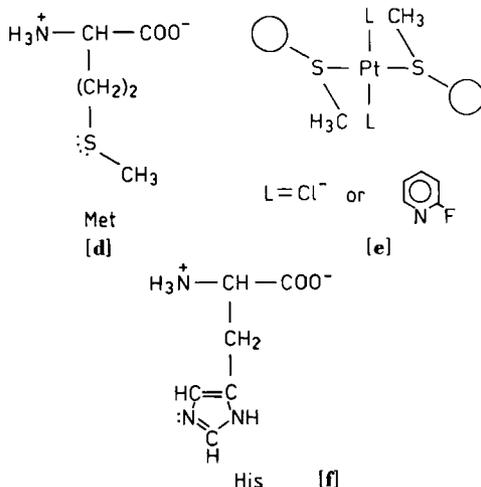
The reaction of cytochrome c from *Candida krusei* and baker's yeast with $[Pt(terpy)Cl]^+$ has also been studied (18), when modification of surface His33 and His39 residues (which are in hydrophilic regions) is observed, but His26 (in a hydrophobic pocket) is largely shielded. The cysteine residue, $HS\text{-}CH_2\text{-}R$ ($pK_a \sim 8.3$), Cys102 in baker's yeast, which is reactive to a

number of reagents, possibly with some perturbation of conformation, is however of low reactivity with $[Pt(terpy)Cl]^+$. This is particularly interesting since, in separate studies with amino acids or small peptides as entering ligands, the complex is completely selective towards cysteine. From a recent crystallographic study on iso-l-cytochrome c, it has been confirmed that the Cys102 is buried and in a hydrophobic region.

Recently it has been demonstrated that incubation of horse cytochrome c with the Rh(II) dimer $[Rh_2(O_2CCH_3)_4]$ for 2 days at pH 7 (but not pH 5) gives the diprotein complex $[Rh_2(O_2CCH_3)_4](cyt\ c)_2$, [g] (19). Attachment

[g]
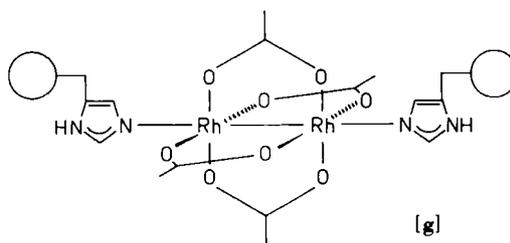
is at a histidine residue, and model complexes $[Rh_2(O_2CCH_3)_4(Im)_2]$ can be prepared, where Im = imidazole, with the Im co-ordinated in the axial position. Since no reaction is observed with tuna cytochrome c, which does not have the His33 residue, it has been concluded that attachment is at this residue on horse cytochrome c. Enhanced stability of the dimer adduct to hydrolysis, as compared to $[Rh_2(O_2CCH_3)_4(Im)_2]$, is attributed to the steric bulk of the protein with the possibility of some H-bonding in addition.

Gold(I) drugs are extensively used in the treatment of rheumatoid arthritis although again the mechanism is not well understood (20). The most commonly used drugs are Myochrisin (gold sodium thiomalate) and Solganal (gold thioglucose), which are administered by weekly or monthly intramuscular injections. The structures of these compounds are not known precisely. The newer drug Auranofin, the gold triethyl phosphine thioglucose complex, can be administered orally (11). It seems likely that the role of the drug is anti-inflamatory and/or anti-enzymatic, and

The Structure of Auranofin

that the body's immune responses are effected in some way. There is some evidence that gold accumulates in the red blood cells. The uptake of Et_3PAu^+ into such cells has been investigated (21). With concentrations of Et_3PAu^+ (up to 9mM) in excess of that achieved in therapeutic applications (25–50μM), interactions with intracellular glutathione, and a second site identified as the cysteine β-93 of hemoglobin, takes place. Excess Et_3PAu^+ also reacts at weaker binding sites (nitrogen or thioether ligands). Comparisons have been made with the binding of gold at weak and strong binding sites identified on serum albumin (22). A facile interprotein gold transfer from gold modified hemoglobin to the -SH containing component of serum albumin has been noted in this work. The application of ^{31}P NMR spectroscopy has been important in these studies.

The use of heavy-atom markers was a major breakthrough in the X-ray structure determination of large bio-molecules. In this process the crystalline material is derivatised for phase determination by a method of isomorphous replacement (23). Procedures involve soaking the crystal in mother liquor containing the heavy-atom marker, or reacting the two together prior to crystallisation. In order to obtain the necessary information, the native diffraction intensities and those obtained from crystals derivatised in at least two unique sites have to be determined.

The reactivities of individual amino acids has been summarised by Petsko and different classes of heavy-atoms defined according to their affinities (23). The most extensively used reagent is $[PtCl_4]^{2-}$ which is capable of binding to methionine, histidine and cysteine residues, none of which are present in large numbers in *metallo*proteins, and in some cases provide unique sites. Some care is required because mother liquors high in $[Cl^-]$ or $[SO_4^{2-}]$ concentrations could impede efficient binding of $[PtCl_4]^{2-}$, which is first converted to aqua and hydroxo forms of neutral or 1+ charge before attaching to the residues indicated (25). Other complexes which will react in the same way are $[Pt(NO_2)_4]^{2-}$, $[Pt(NH_3)_2Cl_2]$ or $[Pt(en)Cl_2]$, where en = ethylenediamine, whereas the inertness of $[Pt(CN)_4]^{2-}$ ensures its retention as a 2– anion which will interact, if at all, with positively charged residues (lysines, arginines and at pH<7 histidines) by electrostatic association. A wide range of mercury compounds including for example ethylmercury phosphate, are known to bind to cysteine or histidine residues (26), and can, because of their structure, readily penetrate into proteins to react with buried side chains. Lanthanides (often samarium because of its large anomalous scattering signal) and uranium compounds will bind at carboxylate residues, although buffers other than phosphate (which will bind to such metals) should be used. The complex ion $K_2[HgI_4]$, which in aqueous solution generates trigonal $[HgI_3]^-$, can bind electrostatically to cations or, because of its flat structure, penetrate into proteins (23).

The strong oxidant osmium tetroxide, which is more stable than RuO_4, is used as a reagent to give syn dihydroxy addition from the less hindered side of a double bond (27). The reagent adds rather slowly but quantitatively to give the intermediate as illustrated [i].

$$-\overset{|}{C}=\overset{|}{C}- + OsO_4 \longrightarrow -\overset{|}{\underset{|}{C}}\overset{O}{\underset{}{\diagup}}\overset{OsO_2}{\underset{}{\diagdown}}\overset{O}{\underset{|}{\underset{}{C}}}- \qquad [i]$$

$$\longrightarrow -\overset{HO}{\underset{|}{C}}\overset{OH}{\underset{|}{C}}-$$

The latter is subsequently decomposed with sodium sulphite in, for example, ethanol. The same reaction can be carried out more economically with hydrogen peroxide using

osmium tetroxide as a catalyst. The procedure finds commercial use in small scale preparations of scarce materials. Osmium tetroxide is highly toxic however, and particularly hazardous to the eyes because of its ready reaction with organic matter to give a black oxide, a property which is utilised in its application as a fixative and stain in electron microscopy. This is probably due to its ability to react with unsaturated fatty acid side chains of lipids (24). Binding of $[OsO_4]^{2-}$ to cis diols has been used in the X-ray analysis of t-RNA where binding is most likely to the 3'-ribose, [ii] (28).

[ii]
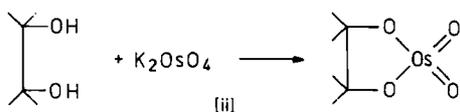
A procedure for detecting genetic changes in new strains of viruses has recently been reported using hydroxylamine and osmium tetroxide (29).

Attachment of pentaammineruthenium(III) to the N-3 position of the imidazole side chain of a histidine residue, [h] in proteins was reported

[h]
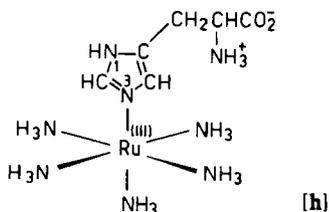
by Matthews and coworkers in 1978 (30). Earlier in the 1970s it had been shown that imidazole and histidine complexes readily form in aqueous solution, and are extremely stable at neutral and acidic pHs. The first studies were with the ribonuclease A protein which has four histidine residues. Three derivatives each containing a single $(NH_3)_5$Ru-histidine complex were synthesised and purified. It was found that sensitivity of the charge-transfer spectrum of one of these derivatives to temperature and urea-induced unfolding could be used to explore conformational changes in the vicinity of the complex (31). It has also been shown that fluorescent energy transfer between tryptophans and a $(NH_3)_5$Ru attached to a histidine

on α-lytic protease and lysozyme can be used to determine inter-residue distances (15.5 and 11.8Å, respectively) (32). At about the same time the preparation and characterisation of a $(NH_3)_5$Ru(III) to histidine-33 derivative of horse cytochrome c was reported (33). This type of attachment to electron transport metalloproteins has now been carried out in a number of cases in order to explore fixed distance (crystallographically defined for the unmodified protein) electron transfer from the attached ruthenium (as Ru(II)), to the active site in its oxidised form, heme Fe(III) in the case of cytochrome c (34). A feature of all these studies is the special affinity of $(NH_3)_5$Ru for histidine in preference to other amino acid residues.

Relevant to these studies is the earlier Taube work, and the observation that both Ru(II) and Ru(III) are inert to substitution. Modification is carried out using a ~50-fold excess of $[Ru(NH_3)_5H_2O]^{2+}$ [iii],

$$[Ru(NH_3)_5H_2O]^{2+} + His\text{-}Protein \rightarrow$$
$$(NH_3)_5RuHis\text{-}Protein \qquad [iii]$$
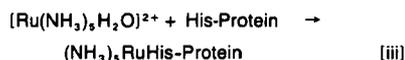
after which the reaction is terminated by chromatographic filtration to remove excess of the ruthenium reagent. The fully oxidised form Ru(III)Fe(III) is obtained by oxidation with $[Fe(CN)_6]^{3-}$ after which further purification (by FPLC) and characterisation is carried out. In order to study the intramolecular electron-transfer process, rapid pulse-radiolysis or flash-photolysis in situ reduction has to be achieved to generate the Ru(II)Fe(III) combination; about 10 per cent reduction is appropriate. The intramolecular electron-transfer process [iv],

$$Ru(II)Fe(III) \rightarrow Ru(III)Fe(II) \qquad [iv]$$

can then be monitored spectrophotometrically. In the cytochrome c case protein concentrations are sufficiently dilute so that there is no contribution from the intermolecular (bimolecular) path [v].

$$Ru(II)Fe(III) + Ru(III)Fe(III) \rightarrow$$
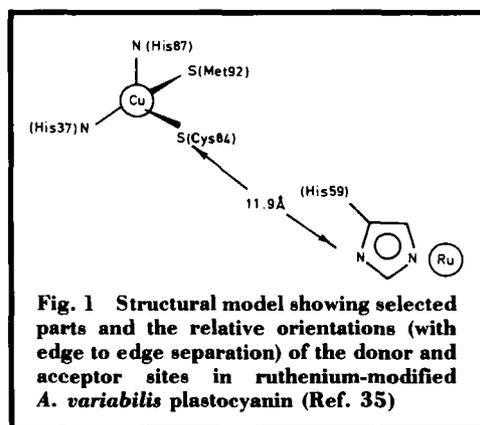$$Ru(III)Fe(III) + Ru(III)Fe(II) \qquad [v]$$
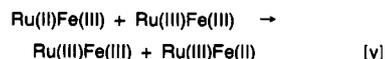
The reduction potentials for the couples

[Ru(NH$_3$)$_5$(His)]$^{2+/3+}$ (o.o8V) and cytochrome c (II)/(III) (o.26V) ensure that there is a positive driving force for [iv]. An interesting variation on this experiment is to attach other ligands to the ruthenium which have much more strongly oxidising couples, for example, [Ru(NH$_3$)$_4$(isn)(H$_2$O)]$^{2+/3+}$ (o.42mV) or [Ru(NH$_3$)$_4$(py)(H$_2$O)]$^{2+/3+}$ (o.33mV), so that a variation on intramolecular electron transfer [vi] can be monitored. This type of study is at present under investigation.

$$Ru(III)Fe(II) \rightarrow Ru(II)Fe(III) \qquad [vi]$$
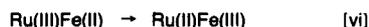
With hind-sight, cytochrome c(II) with its high overall positive charge (estimated as +8 at pH ~ 7) was not in fact a good choice in the first instance for modification with a positively charged complex. The time for modification (24-72h) is substantially longer than that required for acidic negatively charged proteins. Thus with the single copper protein plastocyanin the procedure for [iii] requires only 20 minutes in the case of the acidic plastocyanin (charge 9−) from the green algal source *Scenedesmus obliquus*, but requires approximately 4 hours for the basic plastocyanin (charge 1+) from the blue-green algal source *Anabaena variabilis* (35).
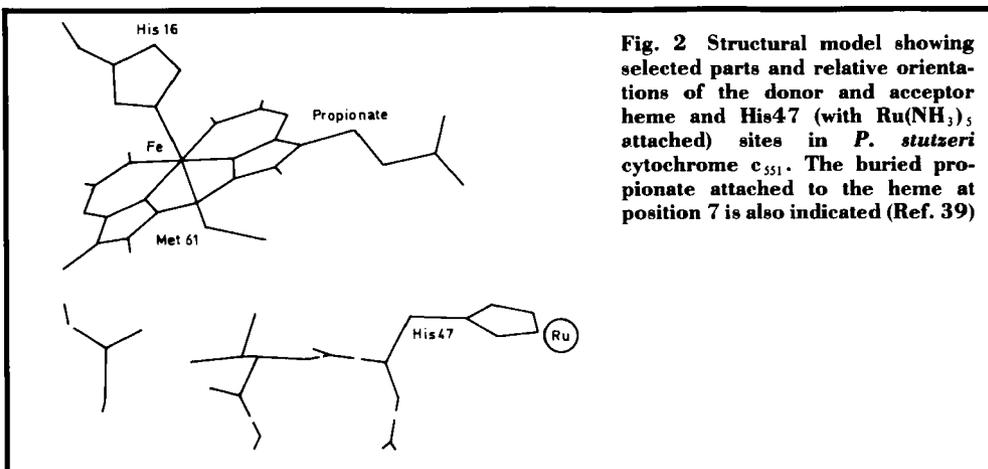
Modification of proteins by the attachment of (NH$_3$)$_5$Ru is not trivial, and extensive characterisation of the products is required for each new protein studied to ensure that attachment is indeed at a histidine residue, and (if more than one histidine) which histidine is involved (35). Techniques used include Inductively Coupled Plasma (ICP) atomic emission spectroscopy to determine the metal content, NMR to demonstrate that the histidine C$_2$H proton resonance is no longer present due to the line broadening effect of paramagnetic Ru(III), and testing with diethyl pyrocarbonate (DEPC) for modification of a histidine (with attendant u.v. spectrophotometric changes) which can no longer occur when ruthenium is present. There is no evidence to suggest that ruthenium attachment is at all disruptive.

Three different types of electron-transport protein have been modified and will be referred

Fig. 1 **Structural model showing selected parts and the relative orientations (with edge to edge separation) of the donor and acceptor sites in ruthenium-modified** *A. variabilis* **plastocyanin (Ref. 35)**

to. Plastocyanin (35) and azurin (36) each have a single copper active site, and utilise Cu(II)/Cu(I) redox states. High-potential iron-sulphur protein (HIPIP) has a cuboidal Fe$_4$S$_4$ cluster which can be either Fe$_4$S$_4$$^{3+/2+}$ (37), while both the cytochromes c and c$_{551}$ contain a heme Fe and have Fe(III)/Fe(II) stable states (38, 39). It should be noted that not all proteins have histidine residues, and that not all histidines are modified by ruthenium. For example in the case of cytochrome c, His33 is readily modified but His26 is not.

Some care is required in defining the distance (d) for intramolecular electron transfer from the Ru(II). For the copper proteins the Cu-S (cysteine) is likely to be the lead-in group, and since there is delocalisation between copper and sulphur the relevant distance is to the S-atom, Figure 1 (35). Similarly there is delocalisation at the imidazole ring of the histidine to which the ruthenium is attached, and the nearest point of the imidazole to the active site is considered relevant. A computer graphics representation for *P. stutzeri* cytochrome c$_{551}$ is illustrated in Figure 2 (39). The direct polypeptide link between His47 and the heme is circuitous in the extreme, and cannot be relevant. Electron transfer to the axially co-ordinated Met61 has been assumed, but transfer to the heme ring, which is strongly electron delocalised, is also possible. For HIPIP, Figure 3, the distance to the nearest point of the Fe$_4$S$_4$ cube, one of the μ_3-sulphido

Fig. 2 Structural model showing selected parts and relative orientations of the donor and acceptor heme and His47 (with Ru(NH₃)₅ attached) sites in *P. stutzeri* cytochrome c_{551}. The buried propionate attached to the heme at position 7 is also indicated (Ref. 39)
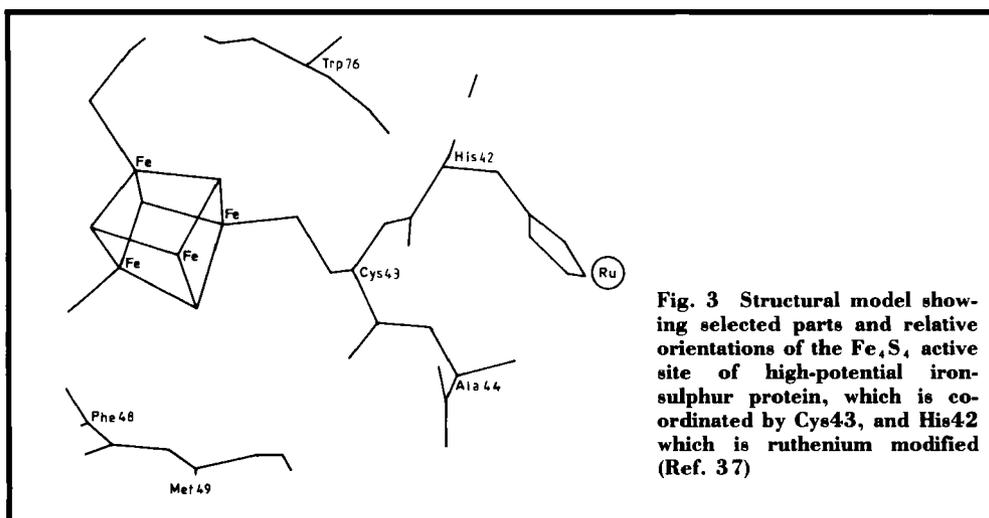
ligands, is considered relevant (37). Of all the studies to date HIPIP is the only protein in which the modified histidine is linked directly by a short polypeptide chain to the active site (Cys43 is co-ordinated to the Fe_4S_4). The through bond distance (saturated bonds!) is 13Å, whereas the through space distance is only 7.9Å. No benefits seem to accrue from this type of attachment.

Distances (d) for electron transfer coincidentally fall into two groups which are close to 12Å and 8Å, respectively, and values for the thermodynamic driving force ($\Delta E°$) lie in a fairly narrow range 180–300mV, see the Table. At

the outset the distance separating the Ru(II) from the metal active site, and the driving force were expected to be the prime rate determining factors. The results obtained clearly indicate that this is not so and that biological electron transfer is far from simple. It is concluded that protein structure and the nature of the intervening polypeptide material must be important. Of current interest is just how influential any intervening aromatic residues might be. Further results from this work are awaited.

Sperm-whale myoglobin, an oxygen binding protein, has also been used by the Gray group to explore factors affecting electron transfer

Fig. 3 Structural model showing selected parts and relative orientations of the Fe_4S_4 active site of high-potential iron-sulphur protein, which is co-ordinated by Cys43, and His42 which is ruthenium modified (Ref. 37)

A Comparison of Rate Constants for Intramolecular Electron Transfer from Ru(II) in Ruthenium-Modified Metalloproteins

Protein	Site of Modification	d (Å)	ΔE° (mV)	k (s⁻¹)	Reference
Cytochrome c (horse)	His33	11.8	180	30,53[a]	38
Azurin (P.a.)	His83	11.8	240	1.9	36
Plastocyanin (S.o.)	His59	10–12	300	<0.26	35
Plastocyanin (A.v.)	His59	11.9	260	<0.08	35
Cytochrome c_{551} (P.s.)	His47	7.9	200	13	39
HIPIP (C.v.)	His42	7.9	270	18[b]	37

[a] Values obtained by flash photolysis and pulse radiolysis, respectively

[b] Values 1 and 13s⁻¹ have been obtained by the Gray group for two HIPIPs modified at His42

reactivity. This protein has four surface histidines His12, His48, His81 and His116 (all at different distances 14.6–22.1Å from the heme Fe) each of which can be ruthenium-modified. Four different singly-modified $Ru(NH_3)_5$ derivatives have been prepared, and in this case a rate constant (k) against distance (d) relationship ln k versus d appears to hold. Unlike the studies with electron transport proteins reorganisation energy requirements at the active site are more significant. This is because metMb has an axial H_2O ligand whereas deoxyMb is five co-ordinate with no H_2O. The myoglobin intramolecular reactions are somewhat slower therefore on this account.

The successful attachment of ruthenium to proteins and various applications that have resulted poses the question: if ruthenium, why not other metals? As far as electron transfer studies are concerned it is of crucial importance that the ruthenium stays attached (it is substitution-inert $t_{1/2} \gg 1$ min) in both the Ru(II) and Ru(III) oxidation states, and that the reduction potentials are of the required magnitude. Another metal which may behave in this way is osmium, but here very little is known of the (II) state solution chemistry. As far as the other platinum metals are concerned Rh(III) and Ir(III) are too inert to be readily attached, and the (II) states are comparatively rare. In the case of palladium and platinum the stable oxidation states (II) and (IV) are separated by two electrons.

Methods for the selective extraction of gold and platinum ions from an aqueous solution at pH>2 with cells or cell extracts of a microorganism (green or blue-green algae) (40) and fungi (41), and subsequent elution with S-donor ligands are being actively explored. This is the subject of a recent U.S. patent (40). Fungi have also be used to remove precious metals from dilute aqueous solutions (41).

Finally, mention should perhaps be made here of the application of bacterial leaching processes in the extraction of certain metals from their ores which in the case of uranium and copper is already being exploited commercially (42, 43). *Thiobacillus ferrooxidans* is for example able to oxidise iron pyrites (FeS) to soluble Fe^{3+} and SO_4^{2-} with very beneficial results (43). The use of other closely related microorganisms, which are active in hot spring regions, in order to make available the metallic gold present in pyrites deposits is currently receiving attention (44).

This short review illustrates the wide range of potential applications of platinum metal compounds centering around their reactivity with a variety of different bio-molecules. Relevant areas stretch from laboratory applications to medicine via the further development of procedures for mining and recovery. Many of the applications make use of specific chemical properties identified in the already well studied and extensive inorganic solution chemistry of these elements.

Additional abbreviations used in the text, without explanation:

dppe = bis(diphenylphosphino)ethylene, EDTA = ethylenediamine tetraacetic acid,
terpy = terpyridine, py = pyridine, isn = isonicotinamide, Mb = myoglobin.

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