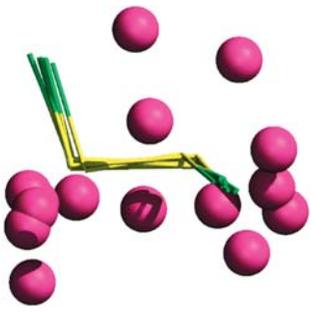

Fig. 12.1.5.6. The relative positions of cystine disulfide bridges (carbon: green; sulfur: yellow) in the parent crystals to the binding of platinum (pink) of $PtCl_4^{2-}$. The cystine side chains have been least-squares fitted, and only those with torsion angles in the range $99.7 \pm 8.3°$ have been used.

octahedral complexes, such as $PtCl_6^{2-}$, with six equivalent covalently bound ligands.

The kinetic and thermodynamic stability of these complexes depends on the protein ligands, buffer, pH and salting in/out agent (Petsko *et al.*, 1978). Anionic groups do not readily react with anionic reagents, such as RS^-, but are attacked more readily by neutral nucleophiles such as *R*SH, *R*-imidazole or RNH_2. The inert cationic group $Pt(NH_3)_4^{2+}$ is most likely to form electrostatic complexes with anionic groups, such as carboxylate. The neutral $Pt(NH_3)_2Cl_2$ molecule, however, can penetrate into hydrophobic areas but requires a stronger nucleophile such as RS^-. In acidic and neutral solutions, $PtCl_4^{2-}$ reacts most commonly with methionine (Figs. 12.1.5.4 and 12.1.5.5), cystine (disulfide) (Fig. 12.1.5.6), N-termini and histidine to form stable complexes. However, methionine reacts faster than histidine. Thus, it is possible to use time as a variable to define specificity. The most popular platinum reagents are listed in Table 12.1.5.4.

In aqueous solution, the square-planar complex $AuCl_4^-$ is hydrolysed to $Au(OH)_4^-$ in about one hour, or in the presence of a protein, reduced to Au(I) by methionine. In ammonium sulfate it probably exists as $AuCl_3(NH_3)$, $AuCl_2(NH_3)_2^+$ and $Au(NH_3)_4^{3+}$. In contrast, $Au(CN)_2^-$ is more stable and normally binds electrostatically. However, on occasions at $pH > 6.0$, the $Au(CN)_2^-$ complex has bound to cysteine residues by nucleophilic displacement reactions.

Osmium resembles platinum in many ways and typically acts as a class B metal. It occurs in all oxidation states from 0 to VIII, but most usually in III, as in K_3OsCl_6; in IV, as in K_2OsCl_6; in VI, as in $K_2OsO_2(OH)_4$; and in VIII, as in osmium tetraoxide, OsO_4. Higher-oxidation-state compounds tend to be reduced to $OsO_2(OH)_2$ in most crystallization solutions and in the presence of ammonia or halide ion they can become further reduced to cationic or anionic complexes, such as $Os(NH_3)_6^{3+}$ or $OsCl_6^{2-}$. Anionic complexes may be substituted by histidine residues at $pH > 7.0$ or bound as ion pairs by histidine at $pH < 7.0$ or protonated amino groups. Cationic complexes tend to bind to negatively charged residues *via* electrostatic interactions.

Iridium is found in all oxidation states from II to VI but commonly exists in III, as in K_3IrCl_6, and IV, as in $(NH_4)_2IrCl_6$. Ir(III) is similar to *rhodium*(III) and is found in a variety of cationic, uncharged and anionic complexes. All Ir(III) complexes are kinetically inert, whereas most anionic complexes of Rh(III) are labile. Ir(IV) is commonly found as the hexahalo complexes IrX_6^{2-} (except iodine), which are also fairly kinetically inert. Cationic [*e.g.* $Ir(NH_3)_6^{3+}$], neutral (*i.e.* $IrCl_3$) and anionic (*i.e.* $IrCl_6^{2-}$) species have proved useful in forming derivatives of protein crystals.

12.1.5.4. *Electrostatic binding of heavy-atom anions*

Positively charged groups of proteins, such as the α-amino terminus, ε-amino of lysine, guanidinium of arginine and imadazolium of histidine, may form ion pairs with heavy-atom anionic complexes. For example, HgI_4^{2-} and HgI_3^- can bind through electrostatic interactions. Anionic metal cyanide complexes tend to be more resistant to substitution and consequently interact electrostatically on most occasions. For example, $Pt(CN)_4^{2-}$ binds at several sites involving lysine or arginine residues in proteins (Fig. 12.1.5.7). $Pt(CN)_4^{2-}$ and $Au(CN)_2^-$ can act as inhibitors by binding at coenzyme phosphate sites.

12.1.5.5. *Hydrophobic heavy-atom reagents*

Since many heavy-atom reagents are hydrophilic, most interactions occur at the protein surface. However, substitution, addition or removal of the non-heavy-atom component(s) of the reagent can alter the hydrophilic–hydrophobic balance and lead to penetration of the core. For example, anionic complexes such as $HgCl_4^{2-}$ and $PbCl_6^{2-}$ are hydrophilic and would not normally enter the protein core, although organometallics, such as *R*HgCl and R_3PbCl (*R* = aliphatic or aromatic), are much more hydrophobic and can do so.

Hydrophobic organomercury compounds of the general formula *R*HgX, where *R* is an aliphatic or aromatic organic group, react with sulfhydryls through displacement of *X*. When *X* is PO_4^{3-}, SO_4^{2-} or NO_3^-, the bond is ionic, making the formation of the cation RHg^+ easier. *R* is often chosen to be a small aliphatic group (*e.g.* CH_3, C_2H_5). However, the presence of a benzene ring enhances the

Table 12.1.5.4. *The five most popular platinum derivatives*

The first column gives the number of times the reagent has been used the analyses included in the heavy-atom data bank.

No.	Compound
287	Potassium tetrachloroplatinum(II)
61	Potassium tetranitritoplatinum(II)
58	Potassium tetracyanoplatinum(II)
57	Dichlorodiammineplatinum(II)
51	Potassium hexachloroplatinum(IV)

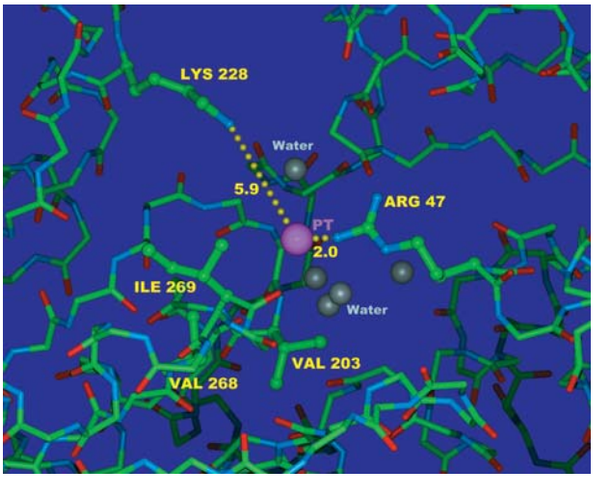

Fig. 12.1.5.7. The binding of $Pt(CN)_4^{2-}$ to aldose dehydrogenase (8ADH).

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stability of the heavy-atom reagent. Careful selection of the *X* group can assist penetration into the hydrophobic core. The hydrophobicity of *X* follows the order

$$PO_4^{3-} < NO_3^- < Cl < Br < I < R.$$

*R*Hg*R* (*R* = aliphatic or aromatic) compounds also bind sulfhydryl residues in hydrophobic regions. The mechanism of reaction of methylphenylmercury with buried sulfhydryl groups may involve fast dissolution in the hydrophobic interior of the protein followed by a slow reaction with neighbouring sulfhydryl residues (Abraham *et al.*, 1983). They are difficult to prepare in aqueous solutions; an aprotic solvent, such as acetonitrile, can improve solubility, but this is not normally a problem in high concentrations of organic components, such as PEG, MPD or ethanol.

Inert gases were first used in the analysis of myoglobin. Schoenborn *et al.* (1965) discovered that the hydrophobic site that bound HgI_3^- also bound a xenon atom at 2.5 atmospheres. They proposed that this may be a general way of producing heavy-atom derivatives of proteins. Recently, there has been increasing interest in this idea, which has now been developed to produce well defined derivatives of a wide range of different proteins. Crystals are subjected to high gas pressures. Xenon requires about 10 atmospheres in order to get saturated binding sites. Krypton binds much less strongly and requires around 60 atmospheres. Since the binding of both inert gases is reversible, it is necessary to keep the protein crystals in a gaseous environment in a specialized pressure cell. Such pressure cells have been developed by Schiltz (1997) at LURE. Xenon binds to hydrophobic cavities, with little conformational change and a retention of isomorphism in crystals. Krypton binds at the same sites as xenon, but since it is lighter and needs higher pressure it has been exploited less by protein crystallographers. However, it has a well defined *K* edge at around 1 Å and so has attractions for multiple-wavelength anomalous dispersion.

12.1.5.6. *Iodine*

In addition to their use in isomorphous replacement, iodine derivatives of crystalline proteins have been prepared as tyrosine or histidine markers to assist main-chain tracing and to act as a probe for surface residues. The order of reactivity towards these reactive residues is

$$\text{Tyr} > \text{His} \gg \text{Trp}.$$

I_3^-, I^-, I^+ and I_2 can be generated by several different methods. An equimolar solution of KI/I_2 or NaI/I_2 in 5% (*v*/*v*) ethanol/water solution is often used to generate the anionic species I_3^- and I^-. An oxidizing agent, such as chloramine T, can be added to KI, typically in a concentration ratio of 1:50; alternatively, polystyrene beads derivatized with *N*-chlorobenzene sulfonamide can be used with NaI. Similarly, the addition of excess KI to ICl or OI^- will generate I_3^-, I^- and I^+. To avoid oxidation of iodine solutions, the pH should be less than 5.0. To avoid cracking the crystals, it may be necessary to increase the iodine concentration very slowly and to wash the derivatized crystals in the mother liquor in order to remove free I_2. Mono- or di-iodination of tyrosines can cause disruption of the protein structure either because of the larger size or the breaking of hydrogen bonds due to lowering of the pK_a of the phenolic hydroxyl.

12.1.5.7. *Polynuclear reagents*

The structure determination of large multicomponent systems such as the 50S ribosomal subunit (Yonath *et al.*, 1986) or the nucleosome core particle (O'Halloran *et al.*, 1987) requires the addition of reagents with a greater number of electrons, preferably in a compact polynuclear structure. Such reagents may be either cluster compounds or multimetal centres having metal–metal bonds.

Polynuclear reagents should preferably be covalently bound to one or a few specific sites, either first in solution or later in the crystals. Spacers of differing length can be inserted into the reagent to increase accessibility. Their low solubility in aqueous solutions can often be overcome by dissolving them in an apolar solvent (*e.g.* acetonitrile). Tetrakis(acetoxymercurio)methane (TAMM) and di-*m*-iododis(ethylenediamine)diplatinum(II) nitrate (PIP) have better solubility in aqueous solutions than other polynuclear heavy-atom compounds.

Polynuclear heavy-atom reagents give an enhanced signal-to-noise ratio in low-resolution MIR studies, but this advantage is offset by the fall-off in scattering amplitude that arises from interference of diffracted waves at higher resolution. In the nucleosome core particle, the scattering reached 50% of its zero-angle value at 7.0 Å, while the relative drop for a single heavy atom was 10% (O'Halloran *et al.*, 1987). Cluster and multimetal reagents that have been successfully employed in protein structure determinations have been reviewed by Thygesen *et al.* (1996).

12.1.6. Metal-ion replacement in metalloproteins

The metal-ion cofactor can sometimes be displaced by dialysis or diffusion by a heavy-atom solution, but usually the cofactor is first removed by a chelating agent (*e.g.* EDTA) or by acidification. These are best carried out on the crystals. Alternatively, the metal

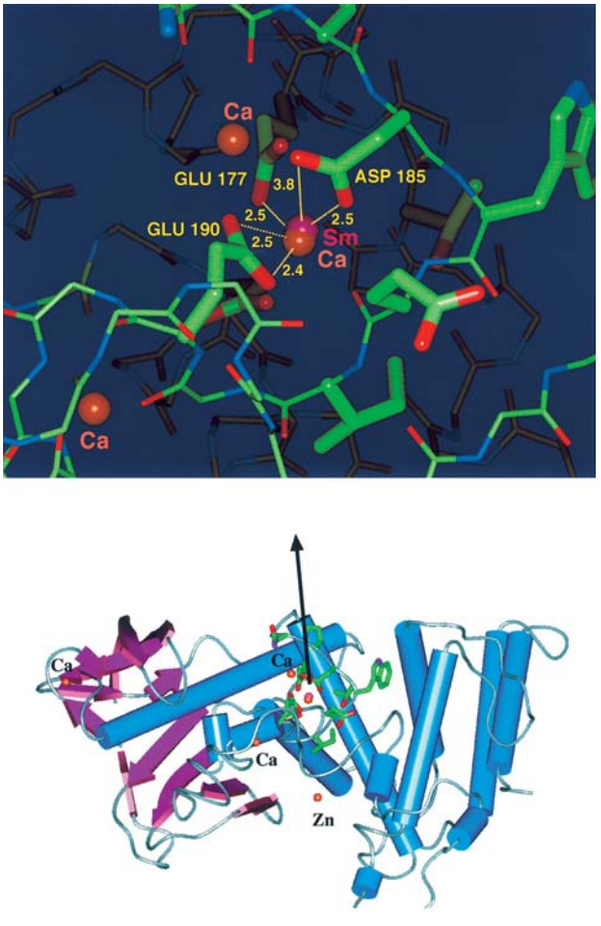

Fig. 12.1.6.1. The displacement of calcium by samarium in thermolysin. The samarium of the heavy-atom derivative is shown superposed on the parent crystal structure.

references