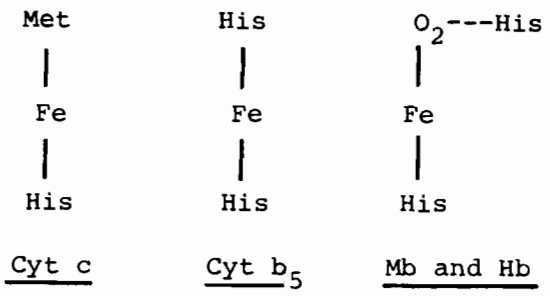

Fig. 1. Axial ligands for iron in haem proteins.

ferric cytochrome into a novel, green high-spin form at pH 7, ambient temperature [1]. The reaction was readily reversed by removal of gold by gel filtration or addition of competing gold-binding ligands. Such a facile spin-state conversion of cytochrome *c* is unusual [2], and we have now extended our studies to other haem proteins with the aim of identifying the critical gold-bonding sites.

The axial ligands for iron in the proteins studied are shown in Fig. 1. The ferric cytochrome b_5 (*Erwinia chrysanthememi*) was predominantly low-spin at pH 7 [3]. Titration with Et_3PAuCl gave rise to an increase in absorption at 610 nm similar to that observed previously with cyt *c* [1], 24 equivalents of gold giving complete conversion to a green high-spin form. The reaction was reversed when gold was removed (gel filtration), but, in contrast to cyt c, too rapid for any kinetic observations by conventional means. From this we concluded that gold attack on either histidine or the haem group itself is involved.

Although Et_3PAuCl rapidly denaturated ferric methaemoglobin, metmyoglobin was fully converted into a green high-spin product by about 30 equivalents of Au at similar haem concentrations (0.07 m*M*). However, in contrast to cytochrome *c*, Et_3PAuCl also reacted with the ferrous proteins. With MbO_2, a two-phase reaction was observed leading to the same high-spin green product as from metMb. HbO_2 behaved similarly but reacted more rapidly [4]. These gold-induced autoxidations appeared to involve release of O_2^-, and that of myoglobin, but not haemoglobin, was reversed by gold removal and $Na_2S_2O_4$ addition.

Direct evidence for structural perturbations around the haem group as a result of gold binding were obtained by a comparison of the paramagnetically-shifted resonances of high-spin ferric myoglobin before and after gold treatment.

Since similar reactions occurred with haemin chloride itself, direct attack of gold on the haem group as well as histidines may be responsible for the observed spin-state changes. Studies on other gold compounds suggest that the Au–P bond remains intact and that N displaces Cl^-. A direct attack by gold on pyrrole nitrogens might be enhanced with a bifunctional gold compound, and indeed we find that only a 12-fold molar excess of $[AuCl]_2$[depe], where depe is (1,2-diethylphosphino)ethane, is required for full spin-state conversion of ferric cyt c.

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The Determination of Binding Sites for Electron Transfer Using Lysine Modified Cytochrome *c* Derivatives

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The heme prosthetic group of cytochrome *c* (MW 12400) is almost completely enfolded by a polypeptide chain of 104 amino acids, leaving the 'front' edge partly exposed to solvent. Although it has been suggested that electron transfer in and out of the protein is via this exposed heme edge [1], the evidence for such a mechanism is often only indirect [2]. At pH 7 cytochrome *c* in the Fe(II) and Fe(III) states carried net charges of +8 and +9 respectively where the distribution of acid residues favours a reaction at the heme edge if the reactant is negatively charged [3]. A positively charged reactant would be guided towards the back of the protein, where the shortest distance to the prosthetic group is 14 Å through the protein. If the exposed heme edge is the only site for electron transfer then positively charged reactants have to overcome a coulombic barrier.

Here we compare the kinetic studies for the oxidation of 4-carboxy-2,6-dinitrophenyl (CDNP) single lysine modified derivatives of horse heart cytochrome *c* [4], with inorganic complexes $Fe(CN)_6^{3-}$ and $Co(phen)_3^{3+}$ [5, 6], and the proteins parsley plastocyanin PCu(II), *Pseudomonas aeruginosa* azurin ACu(II) [7], *Rhus vernicifera* stellacyanin SCu(II), and *Anabaena variabilis* plastocyanin. At pH 7 these proteins have approximate charges of −7, −1, +7 and

+1 respectively. None of the oxidants are physiological partners for cytochrome *c*. Eight cytochrome *c* derivatives have been investigated with modifications at lysines 7, 13, 25, 27, 60, 72, 86 and 87. First-order rate constants were obtained at I = 0.10 *M* (NaCl) with the oxidant always in large excess. The influence of the modified residues on the bimolecular rate constants defines the protein molecular surface involved.

With the inorganic 3– and 3+ oxidants at pH 7.2 the site for electron exchange is the solvent accessible edge of the heme prosthetic group or a closely related structure on the front surface of the molecule. The reaction with $Fe(CN)_6^{3-}$ is most strongly influenced by modification of lysine 72 (3.6-fold decrease in rate constant), a residue to the left of the exposed heme edge. This same locality has been defined from NMR studies as a region where interaction of cytochrome *c* with $Fe(CN)_6^{3-}$ occurs [8]. However, it is the region around lysine 27, to the right of the heme edge, which is most influential in the reaction with $Co(phen)_3^{3+}$ (7.3-fold increase compared to native).

With the blue Cu proteins, the interacting domain on cytochrome *c* is again located at the front surface of the protein and encompasses the solvent accessible exposed edge of the heme group. The rate constant for the reaction with parsley plastocyanin (1.5×10^6 M^{-1} s^{-1}) decreases by half (0.75×10^6 M^{-1} s^{-1}) for the lysine 13 derivative, which is the most influential modification. Conversely the reaction with azurin (–1 charge), which was studied at pH 6.1 and 8.6 (at either extremity of a pH transition), gave rate constants 6.6×10^3 M^{-1} s^{-1} at pH 6.1 and 4.0×10^3 M^{-1} s^{-1} at pH 8.6, which increase on modification to 4.1×10^4 M^{-1} s^{-1} (pH 6.1) and 2.7×10^4 M^{-1} s^{-1} (pH 8.6) for the most influential modification at lysine 72. The reaction with cytochrome *c* occurs at a negative site on plastocyanin, whereas azurin behaves as a positively charged reactant, and displays very similar characteristics to stellacyanin (+7) and *A. variabilis* plastocyanin (+1). Thus with the latter at pH 7.0 the rate constant of 2.5×10^3 M^{-1} s^{-1} for native increases on modification at lysine 72 to 2.2×10^4 M^{-1} s^{-1} for stellacyanin and at pH 7.5 a value of 7.3×10^4 M^{-1} s^{-1} for *A. variabilis* plastocyanin increases to 2.6×10^5 M^{-1} s^{-1} for the lysine 72 modification.

The differences in reactivity patterns for the blue Cu proteins probably reflect the different distributions of charged and uncharged residues on the surface of the proteins, which result in different charge as well as other secondary interactions. They do not necessarily indicate the use of different sites on cytochrome *c* as is implied with the inorganic oxidants. Indeed the reactions of the blue Cu proteins with cytochrome *c* are non-physiological, and these proteins have not been subjected to selective evolutionary forces that would have resulted in favourable co-adaptation of surface domains relevant to electron transfer. Thus the bimolecular rate constants for these reactions are small compared to those involving electron exchange with the natural redox partners for cytochrome *c*. Also we note that the lysine modification effects reported here are small compared with the massive effects observed for the physiological partners [9].

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A Comparative Study of Free and Polymer Immobilized Hemin Catalysts of Oxidation of Cystein

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Iron porphyrins attached to side ionizable groups of ion exchange polymers (styrene–divinyl benzene matrix) may be considered as model compounds of heme proteins.

We studied the conditions of immobilization of hemin (iron(III) protoporphyrin IX chloride) on different ion exchange polymers and the catalytic activity of bound and free hemin species in the oxidation reaction of cystein (RSH):

$$4RSH + O_2 \rightarrow 2RS{-}SR + 2H_2O$$