THE STRUCTURAL FLEXIBILITY OF FERRIC CYTOCHROME *c*

Regulation of the spin-state equilibrium by an anti-arthritic gold(I) compound at neutral pH

Gabriel OTIKO and Peter J. SADLER

Department of Chemistry, Birkbeck College, University of London, Malet Street, London WC1E 7HX, England

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1. Introduction

In the eukaryotic mitochondrial respiratory chain, cytochrome *c* transfers electrons from a reductase complex to cytochrome oxidase, the terminal component which reduces molecular oxygen to water. Cytochrome *c* is a haem protein containing about 103 amino acids. The haem group is covalently attached to the protein via thioether linkages to one of its edges from Cys 14 and Cys 17, and the iron itself is further coordinated in its fifth and sixth positions to S from Met 80 and N from His 18 [1]. With such a ligand field the spin-state equilibrium (1) lies almost entirely toward the low-spin form:

$$\underset{\text{low-spin}}{Fe^{3+}\text{-Cyt } c\ (S = 1/2)} \rightleftharpoons \underset{\text{high-spin}}{Fe^{3+}\text{-Cyt } c\ (S = 5/2)} \quad (1)$$

This is characterised particularly by a weak electronic absorption band at 695 nm usually assigned to an Fe^{3+}–S (Met) charge-transfer transition [2]. Successful attempts to bleach this band and hence to rupture the Fe–S bond usually require extreme conditions: high or low pH [3], high temperature [4], or covalent modification [1]. When structural disruption occurs, other strong-field ligands can become available to Fe within the haem pocket, and modified cytochrome *c* can remain low-spin.

We report here a facile conversion of low-spin Fe^{3+} cytochrome *c* to the high-spin form, at neutral pH and ambient temperature, through association with the anti-arthritic gold compound [5] triethylphosphine gold(I) chloride, Et_3PAuCl. The kinetics and extent of binding have been followed by conventional electronic absorption spectroscopy, and reversibility demonstrated by Sephadex chromatography and addition of competing, gold-binding ligands. Nitrogen of His and sulphur of thioethers (Met and Cys) are implicated as gold binding sites.

These studies confirm that Fe^{3+} cytochrome *c*, in contrast to Fe^{2+} cytochrome *c*, has the structural flexibility to accommodate spin-state changes readily. These may be involved in gating electron flow in the mitochondrial electron transport chain.

2. Materials and methods

Horse heart cytochrome *c* type VI was purchased from Sigma. It was fully converted to Fe^{3+} by treatment with excess $K_3Fe(CN)_6$, or to Fe^{2+} with excess $Na_2S_2O_4$. These reagents were removed by passage down a Sephadex G-25 column with 0.1 M phosphate buffer (pH 7) as eluant. Solutions were standardised using known extinction coefficients.

Gold compounds were kindly supplied by Smith Kline and French Labs. (Philadelphia).

Electronic absorption spectra were recorded on Perkin Elmer 402, or Pye Unicam SP8000 spectrometers, in 1 cm or 1 mm (Soret band) cells. Checks on the infrared bands were also made on a Cary 14 instrument. In a typical experiment for kinetic measurements, 15 μl of a 143 mM solution of Et_3PAuCl in MeOH was added to 2 ml cytochrome *c* in phosphate buffer (pH 7) and the solutions were left to stand for 24 h before their spectra were recorded. Appropriate controls containing MeOH alone were also studied but there was no effect on the spectra at these concentrations. MeOH binding is known to be very weak [6].

Conductivity measurements were made at 20°C on

a Griffin conductivity bridge equipped with a Chandos conductivity cell (Pt black electrodes). Electron spin resonance spectra were recorded on an X-band Varian spectrometer at 9.24 GHz equipped with a liquid helium dewar (10 K) with the grateful help of Dr A. J. Thomson and co-workers at the University of East Anglia. Et_3PAuCl in 0.1 ml of MeOH was added to 1 ml of a 2 mM solution of Fe^{3+} cytochrome *c* in phosphate buffer, giving a 14-fold excess of drug. Some drug precipitation occurred and the spectrum of the clear supernatant was recorded.

3. Results and discussion

The electronic absorption spectra shown in fig.1 illustrate the time-course of the reaction between Fe^{3+} cytochrome *c* and Et_3PAuCl. Isosbestic points were observed at 402, 460, 522 and 546 nm indicating that at least two independent species are involved in the reaction. It will be shown that these are low-spin Fe^{3+} cytochrome *c*, the starting material, and high-spin Fe^{3+} cytochrome *c*, the product. A plot of $\log (A_e - A_t)$ versus time was a straight line (fig.1). From this a pseudo first-order rate constant of 8×10^{-3} s^{-1} (20°C) was calculated. A_e and A_t are the absorbance values (at 596 nm) at equilibrium and time *t*, respectively.

A series of solutions containing Fe^{3+} cytochrome *c* and a 5–50-fold excess of Et_3PAuCl were prepared and left to reach equilibrium. It was apparent that the new species was fully-formed in the presence of >40

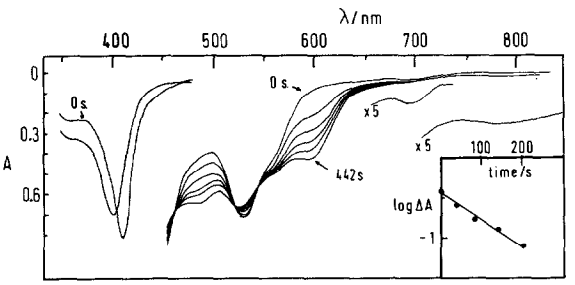

Fig.1. Electronic absorption spectra of 0.064 mM Fe^{3+} cytochrome *c* in the presence of a 20-fold excess of Et_3PAuCl (0.1 M phosphate buffer (pH 7), 20°C, 1 cm cell) at various times after mixing (0, 37, 82, 144, 202 and 442 s at 596 nm, scan rate 6.2 nm/s). From 350–450 nm and beyond 700 nm, only the initial and final spectrum are shown. The spectra of the Soret band are from a 0.069 mM solution containing a 25-fold excess of drug (1 mm cell).

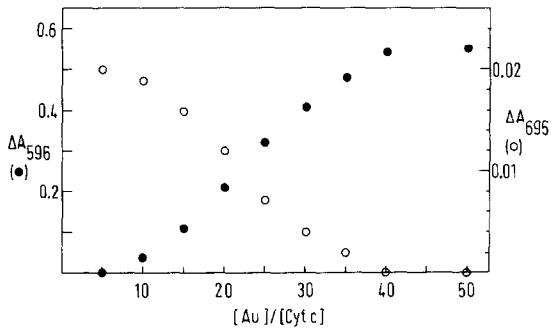

Fig.2. The decrease in A_{695} (low-spin band) and increase in A_{596} (high-spin band) in a series of mixtures of Fe^{3+} cytochrome *c* (0.069 mM) and Et_3PAuCl at equilibrium.

equiv. Au. The increase in A_{596} for example, paralleled the decrease in intensity of the 695 nm band, fig.2. 100% conversion could also be achieved by heating a solution containing 20 equiv. to 55°C. However, this was not fully reversible on cooling.

The spectrum of the new species is characterised by maxima at 486, 524, 550 and 596 nm. The Soret band shifts from 409–400 nm and a new, very broad band appears in the infrared region at ~790 nm. These features are consistent with the formation of high-spin Fe^{3+} cytochrome *c*. Absorption spectra of high-spin haem proteins usually contain four diffuse bands in the region 450–650 nm and a weaker band at 700–1100 nm [7]. Our spectrum is also similar to that reported for (Met 80 sulphoxide)–Fe^{3+} cytochrome *c* [8], including the shift of the Soret band. It is noteworthy that (Met 80 sulphoxide)–cytochrome *c* is still catalytically active toward cytochrome oxidase, but additional photooxidation of His 18 destroys the infrared band and the activity [8]. The possibility must be considered that the high-spin form can still contain a Met S–Fe^{3+} bond, although now weakened.

EPR experiments verified that a high-spin Fe^{3+} haem species was formed, with signals at $g = 2.0$, and rhombic, $g = 6.44$ and 5.12, and axial, $g = 6.23$ and 5.82, components in the perpendicular region (fig.3). They display a curious resemblance to spectra reported for cytochrome oxidase [9]. The high-spin signals reverted completely to those of the low-spin form ($g = 3.07$ and 2.23) after passage of the sample down a Sephadex G-25 column. This was also true for the electronic absorption spectrum. Low molecular weight drug would be removed on the column.

A large increase in magnetic susceptibility of cyto-

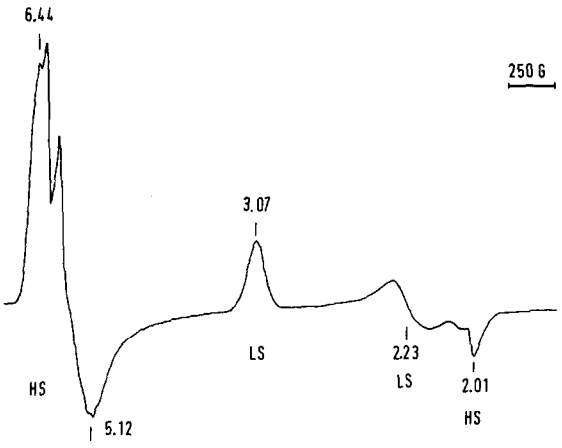

Fig.3. X-Band EPR spectrum of a 2 mM solution of ferric cytochrome *c* in the presence of excess Et_3PAuCl at 10 K. Peaks due to the high-spin (HS) and low-spin (LS) forms are marked.

chrome *c* on addition of Et_3PAuCl was observed by the NMR method. A magnetic moment of ~5.9 BM was calculated. This is about the value expected for a high-spin Fe^{3+} haem ($S = 5/2$).

We have been able to implicate N of His and S of thioethers (Met, Cys) as possible gold binding sites on cytochrome *c* through experiments in which the spin-state conversion was reversed by addition of other competing ligands. We noted that Et_3PAuCl caused no conversion of low-spin cytochrome *c* to the high-spin form when the reaction was carried out in 50 mM Tris buffer (pH 7). A direct reaction between Tris free base, $H_2N{-}C{-}(CH_2OH)_3$ and $ClAuPEt_3$ to give $[Et_3PAu\,NH_2\,C(CH_2OH)_3]^+$ was suspected because the drug remained soluble even when present in large excess (60 X). Seven equivalents of Tris (free base) with respect to Au were sufficient to reverse fully the spin-state conversion in a solution containing 0.069 mM cytochrome *c* and 2.3 mM Et_3PAuCl (100% high spin). In view of this finding, Tris buffer should be avoided in studies of the biochemical pharmacology of Et_3PAuCl.

Reversal was also complete on addition of 1.5 equiv. *N*-methylimidazole, or 0.7 equiv. L-histidine. Et_3PAuCl is readily solubilised to 0.1 M in water alone on adding 2 equiv. *N*-methylimidazole, and direct N binding to Au is indicated by shifts of the ^1H NMR resonances. Since conductivity measurements indicate formation of a 1:1 electrolyte, we assume that $[Et_3PAu\text{-}(NMe\text{-}Imid)]^+$ is formed. *N*-Methylimidazole is in fast exchange between the free and bound forms on the NMR timescale.

Conductivity measurements suggest that Et_3PAuCl is ~20% hydrolysed, eq. (2), in aqueous MeOH solutions at concentrations used in cytochrome *c* experiments:

$$Et_3PAuCl + H_2O \rightleftharpoons [Et_3PAuOH_2]^+ + Cl^- \qquad (2)$$

There are three histidines in horse cytochrome *c*. His 33 is readily modified by chemical reagents such as bromoacetate without causing a haem perturbation [1], whereas His 26 and His 18 (haem ligand) are usually less accessible. Gold binding at Cys 17 may enhance the accessibility of His 18.

We have shown [10] that Au(I) also has an affinity for Met S atoms and find here that 6 equiv. *N*-acetyl-L-methionine (with respect to Au) reverse fully the spin-state conversion. Shifts of the ^{31}P NMR resonance of Et_3PAuCl can be observed on titration with NAcMet. Met 65 and Cys 17 are both exposed on cytochrome *c*, whereas Met 80 and Cys 14 are buried. It is possible that binding at Cys 17 may disturb the coordination of the neighbouring haem ligand His 18 and open up the haem crevice sufficiently to allow binding at Met 80. Binding to Cys 17 is involved in the haem cleavage reaction induced by Ag(I) at low pH [11]. Chemical modification of Met 65, on the other hand, does not usually perturb the haem.

Au–Cl bond cleavage is clearly important in promoting cytochrome *c* spin-state changes. A 40-fold excess of NaCl (with respect to Au) causes complete reversal, and neither Et_3PAuBr (15 X -solubility problems at higher levels) nor (2,3,4,6-tetra-*O*-

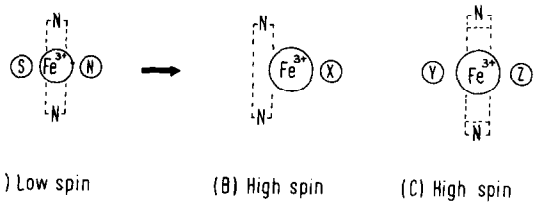

Fig.4. Fe is 6-coordinate in low-spin Fe^{3+} cytochrome *c* and situated in the porphyrin plane (A). Conversion to high-spin may lead to its displacement from the plane by up to 0.5 A (larger ion) and to 5-coordination, as in (B), or to radial expansion of the porphyrin core whilst remaining in-plane (C) [13,14]. Significant changes in doming of the porphyrin may also occur. X is likely to be N from His 18. Y and Z have to be weak-field ligands, e.g., H_2O or weakly-bonded Met.

acetyl-l-thio-β-D-glucopyranosato-*S*)-triethyl-phosphine gold (auranofin, 15 ×) were effective. Au(I)–Br and Au(I)–SR bonds are stronger than Au(I)–Cl.

No spectral change was observed when Et_3PAuCl was added to low-spin Fe^{2+} cytochrome *c*. The Fe–S bond is stronger in the reduced form and the structure is much tighter [1,12].

A spin-state change in Fe^{3+} cytochrome *c* would be expected to lead to significant structural changes of the type summarised in fig.4. They might be used to transmit effects to other components in the electron transport chain, as part of a gating mechanism. The redox potential would be lowered. The role of Cu(I) in cytochrome oxidase has yet to be fully defined, and, in view of similarities in Au(I) and Cu(I) chemistry, it is conceivable that Cu(I) could mimic the behaviour of Au(I) described here.

A detailed NMR study of cytochrome *c* to identify the gold binding sites is in progress.

Acknowledgements

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