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The Nature and Functional Significance of the Fe–Cu Binuclear Centre in Cytochrome *c* Oxidase

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Cytochrome *c* oxidase catalyses the overall reaction shown below

$$4c^{2+} + 4H^+ + O_2 \rightarrow 2H_2O + 4c^{3+}$$

where c^{2+} and c^{3+} are, respectively, the reduced and oxidised forms of cytochrome *c*. The enzyme contains four prosthetic groups; two haem iron moieties and two copper atoms [1] and a variety of spectroscopic studies have led to the conclusion that two of the four metals, one iron and one copper are involved in a substantial magnetic interaction [2]. This binuclear centre includes the haem of cytochrome a_3 and one of the coppers, known variously as undetectable copper; Cu_u, Cu_B or Cu_{a3}. A shared ligand is thought [3] to mediate the magnetic interaction and it has long been known that cytochrome a_3 is the site of interaction with the respiratory poisons CO and CN^- and, by analogy, the substrate molecule, O_2. The nature of the binuclear centre, its coupling and functional significance is clearly of fundamental importance and we have attempted to explore this through the use of a range of spectroscopic approaches, both static and kinetic.

Examples of ligand occupancy at each metal site in the binuclear complex are available. Carbon monoxide forms a well characterised complex with ferrous a_3 [4] and has been suggested to bind also to $copper^{I}_{a3}$ [5]. Nitric oxide binds to $copper^{II}_{a3}$ and reacts with ferric a_3 as a reductant and ligand, behaving in this respect like carbon monoxide, to produce a mixed valence ligated complex of the enzyme. Cyanide reacts with both the oxidised and reduced iron of a_3 blocking, in the former complex, any subsequent reduction of the a_3 iron [4]. The low temperature M.C.D. spectrum between 700 and 2000 nm of the oxidised cyanide bound enzyme shows three features at 790 nm, 1564 nm and 1946 nm and the magnetic saturation curves for each of these suggests that they arise from three distinct paramagnetic centres. The features at 790 nm and 1564 nm are due to the E.P.R. detectable components of the enzyme Cu_a and haem *a* respectively. The peak at 1946 nm has the shape expected for the porphyrin → Fe^{III} charge transfer band of a low spin ferric haem and we consider this strong evidence for its identification as haem a_3-CN, although the extremely long wavelength of the peak of this band makes it unique among the near I.R. spectra of low spin ferric haems so far reported. The magnetization characteristics suggest a ground state for haem a_3-CN of S = 1 and taken together with the shape indicate that this haem, in the cyanide inhibited enzyme is low spin ferric and ferromagnetically coupled to Cu^{II}_{a3} to give an effective ground state spin S = 1. Treatment of the enzyme with dithionite results in the formation of a partially reduced state in which all the metal centres except a_3-CN are reduced and diamagnetic. The haem a_3-CN becomes E.P.R. detectable with a resonance at $g = 3.58$ and the 1946 nm band is lost, to be replaced at 1500 nm by a typical low spin ferric CT band whose magnetization characteristics originate from a paramagnet with $g_z = 3.58$. This result taken together with the observations on the fully oxidised cyanide inhibited enzyme can be explained if CN^- forms a bridge between haem a_3 and Cu^{II}_{a3}, thus: Fe^{III}–CN–Cu^{II}_{a3}.

We have further investigated this species using the mixed ligand complex formed by fully oxidised cytochrome oxidase in the presence of cyanide and nitric oxide. Fully oxidised cyanide bound cytochrome oxidase reacts with NO to give a species with a g value at 3.44 in addition to 3.03 (ferric haem *a*) and 2.0 (copper *a*). On illumination at 10 K, the $g = 3.44$ signal is abolished but returns on warming to 50 K, behaviour not dissimilar to that of the NO complex of the fully reduced enzyme. The $g = 3.44$ signal is not due to partial reduction of the enzyme since the *g* value arising from partially reduced cyanide bound enzyme is at 3.58 and this species is not photosensitive. Low temperature M.C.D. spectroscopy shows three transitions 790 nm ($copper^{II}_{a}$) 1564 nm (low spin ferric *a*) and 1790 nm, a band which disappears on illumination at 10 K to be replaced by the characteristic band at 1946 nm assigned to low spin ferric a_3 CN coupled to $copper^{II}_{a3}$. The M.C.D. saturation curve obtained at 1790 nm identifies this feature with the $g = 3.44$ feature seen in E.P.R. and suggests that the 1790 nm band represents haem a_3^{3+} CN influenced by the NO bound copper a_3 (Cu^+NO^+) through the coupling which persists in this particular derivative of the enzyme.

The reaction of mixed valence enzyme *i.e.* of the reduced binuclear centre with oxygen has been followed using flow flash photolytic procedures and provides evidence at room temperature for the occurrence of Cpd A (oxyferro species) only previously seen at very low temperatures.

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Europium(III) Laser Luminescence Excitation Spectroscopy of Calcium-Modulated Proteins: Parvalbumin and Calmodulin

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Tripositive lanthanide ions, symbolically Ln(III), have been used for more than a decade to probe calcium-binding sites in proteins [1]. Efforts in this laboratory [2] have been devoted to the development of methods to exploit the luminescence properties of certain of the lanthanide ions [3, 4], notably Eu(III) and Tb(III), in the study of this class of protein. Central to our research is the use of a tunable, pulsed dye laser to excite directly the metal ion levels. The Eu(III) ion, with its nondegenerate ^7F_o ground state, is particularly useful as a probe species. Excitation spectroscopy of the $^7F_o \rightarrow {}^5D_o$ transition in the 578–581 nm region (monitored via the $^5D_o \rightarrow {}^7F_2$ emission at ~612 nm) has been useful in detecting and characterizing individual, distinct Eu(III) ion binding sites in proteins [2, 5]. The observation of more than one peak in such an excitation spectrum is unambiguous evidence for the existence of more than one type of binding site. In addition, sites can be characterized via their Eu(III) (or Tb(III)) excited state lifetimes in H_2O and D_2O solution in order to determine how many water molecules are coordinated to the ion at each site [6]. Laser spectroscopic methods can also be used to measure distances between Ca(II) ion binding sites by monitoring Förster-type nonradiative energy transfer between emissive (Eu(III), Tb(III)) and absorbing (Pr(III), Nd(III), Ho(III), Er(III)) ions occupying nearby sites [7–9].

Calcium-modulated proteins are an important class of macromolecule found in the cytosol [10]. These proteins, which include calmodulin, troponin C, myocin light chains, parvalbumin and intestinal calcium-binding protein, all bind Ca(II) ions with pK_d values ($-\log K_d$) between 5.0 and 6.5 in the presence of millimolar concentrations of Mg^{2+}. Ln(III) ions bind even more tightly and spontaneously replace bound Ca(II) ions. Two members of this class of protein, namely parvalbumin and calmodulin, are the objects of the present study.

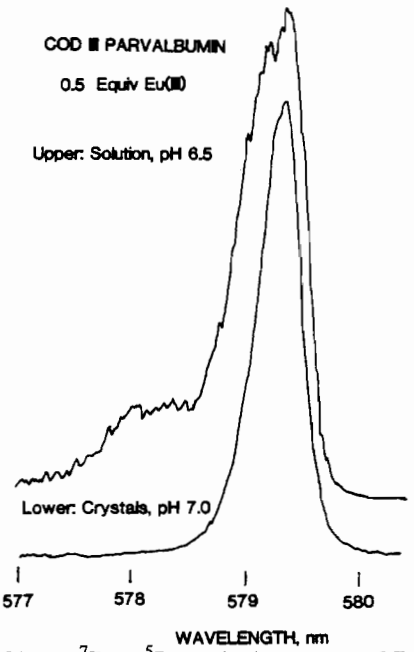

Fig. 1. $^7F_o \rightarrow {}^5D_o$ excitation spectra of Eu(III) in parvalbumin in solution and crystalline states.

Results and Discussion

Parvalbumin. This protein binds two Ca(II) ions strongly. Earlier work in this laboratory [7, 11] has shown that at pH 4.0 the principal CD and EF sites are filled simultaneously during a titration with Eu(III), while at pH 6.5 a third site is occupied as well. Excited state lifetime results suggest that one water molecule is coordinated to the CD and EF Eu(III) ions while three are involved at the third site. The occupancy of this third site was shown to be responsible for the 'quenching' of Tb(III) luminescence after more than 1.5–1.8 equivalents of this ion have been added. The solution-state results are in apparent disagreement with X-ray diffraction studies [12], which show that if parvalbumin is crystallized in the presence of less than one equivalent of Tb(III) only the EF site is occupied while with more Tb(III) present both CD and EF sites are filled. No evidence for a third metal ion binding site was found in the X-ray study [12]. Figure 1 shows the $^7F_o \rightarrow {}^5D_o$ excitation spectrum of a parvalbumin solution (upper trace) containing 0.5 equivalents of Eu(III) which provides evidence for the occupation of all three sites. This solution, when dialyzed against 2.8 *M* $(NH_4)_2SO_4$ buffer at pH 7.0, yielded crystals whose excitation spectrum is shown in the lower trace. Only a single sharp peak indicative of the sole occupancy of the EF site is