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Why do *c*-type cytochromes exist? – Reprise

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An earlier paper (Wood, P.M. (1983) FEBS Lett. 164, 223–226) proposed that the covalent links that characterize *c*-type haem originated in order to prevent the haem being lost into the external medium. This is discussed in relation to Gram-negative and Gram-positive bacteria, cyanobacterial thylakoids and eukaryotes. The practical application is for unravelling complex electron transfer chains: *c*-type cytochromes may be assumed to be absent from the cytoplasm. Sulphate reducing bacteria provide the only confirmed exception to this rule. Examples of non-covalent haem at risk of being lost are considered. Analogies are drawn with flavin and pyrroloquinoline quinone as prosthetic groups.

The hypothesis

In 1983 I published a paper with the above title [1]. I shall summarize my line of argument and make comments in the light of more recent research.

The distinguishing feature of *c*-type cytochromes is a haem group as in *b*-type cytochromes or globins, but with covalent bonds between the two vinyl groups of the haem and cysteine side-chains of the protein. A comparison of proteins with CO-binding *b*- or *c*-type haem provides no evidence that the covalent bonds confer any novel properties on the haem [2]. Among other haem proteins, covalently attached haem is very unusual.

One generalization concerns the location of *c*-type cytochromes in bacteria. With very few exceptions (discussed below), soluble cytochromes *c* in bacteria are periplasmic. Membrane cytochromes *c* have the haem near the periplasmic face of the membrane, as in cytochrome c_1 or the tetrahaem reaction-centre cytochrome of certain photosynthetic bacteria [3].

Any non-covalently bound prosthetic group has a finite dissociation constant, values up to 10^{-9} M being reported for protohaem [4]. Inside the cell there is a finite concentration of free haem, so reassociation can occur, as in biosynthesis. However, pores in the outer membrane of Gram-negative bacteria have an exclusion limit above the size of free haem [5]. This led me to propose that the different classes of *c*-type cytochromes originated as proteins located in the bacterial periplasmic space, or on the periplasmic face of the cytoplasmic membrane [1]. In these locations, covalent bonds between haem and protein prevented the haem from being lost into the surrounding medium.

The practical application of this hypothesis lies in unravelling complex electron transfer chains: *c*-type cytochromes may be assumed to be absent from the cytoplasm. For sulphate reducers, see below.

Gram-positives, cyanobacteria and eukaryotes

A few soluble cytochromes *c* are known from Gram-positive bacteria [6]. Gram positives have no outer membrane and hence no periplasm as a discrete compartment. This has led to the suggestion that a soluble protein must be cytoplasmic – otherwise it would diffuse away [7]. However, there is evidence that soluble proteins can be retained on the outer face of the cytoplasmic membrane by non-covalent forces [6]. This location has been demonstrated for a Class I *c*-type cytochrome in *Mycobacterium phlei* [8].

The *c*-type cytochromes of eukaryotes and cyanobacterial thylakoids are in locations where haem tethering would not seem to be important. These proteins are homologous to cytochromes found in simpler organisms [1,3]. They are never located on the same face of the membrane as the proton-translocating ATP-synthase. The thioether bonds in *c*-type cytochromes are important in maintaining the correct conformation of the haem relative to the polypeptide. To dispense with them and retain the same molecular properties would involve much rebuilding of the protein, and hence is an unlikely

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evolutionary step. The addition of free haem to apomyoglobin allows folding into an active conformation [9]. This result has never been obtained with an apocytochrome *c* [3]. (See, however, the comments on cytochrome b_2 below.)

Cytoplasmic c-type haem in sulphate-reducing bacteria

In my paper I discussed some apparent exceptions [1]. The only recent work on soluble bacterial cytochromes *c* pointing to a cytoplasmic location is for sulphate reducers. In such organisms the tetrahaem cytochrome c_3 is periplasmic, and this is the general location for the monohaem cytochrome *c*-553 [10,11]. The N-terminal residue of these cytochromes is Ala or Val, followed by a limited range of amino acids in position 2 [10]. These sequences are consistent with an N-terminal leader peptide. However, an octahaem cytochrome c_3 has not been found among periplasmic proteins [11]. The N-terminal sequence of this protein from *Desulfovibrio baculatus* is Glu-Thr, which is not typical for a signal peptidase cleavage site [10].

The octaheme cytochrome c_3 is a dimer of identical subunits. The N-terminal region shows sequence homology with tetraheme cytochrome c_3 once the first haem attachment site appears [12]. Thus the two proteins diverged from a common ancestor. The present hypothesis would require that the ancestral cytochrome was periplasmic. Gene duplication permitted divergence of a slightly modified form, which was found useful as a cytoplasmic electron carrier. The retention of the covalent links would be for the same reasons as in eukaryotes or cyanobacterial thylakoids.

For *D. baculatus*, but not for other species, the N-terminal sequence of monohaem cytochrome *c*-553 is not consistent with a previous signal peptide. LeGall and Peck [10] propose a cytoplasmic location, not yet confirmed.

The biosynthesis of *c*-type haem requires a special enzyme, cytochrome *c* haem lyase [13]. The enzyme achieves the correct union of each vinyl with the corresponding thiol and selects the correct chirality at each thioether out of the possible isomers. In some bacteria the haem lyase has to recognise ten or more haem attachment sites.

The dual location for *c*-type haem in sulphate-reducing bacteria raises intriguing questions about the location and mechanism of haem attachment.

Non-covalent haem at risk of being lost

There are many examples of the converse situation – haem without covalent links in the bacterial periplasm, or released into the external medium.

White-rot fungi such as *Phanerochaete chrysosporium* use haem-containing peroxidases to assist in lignocellulose breakdown [14]. These extracellular proteins normally operate in a confined space, as the mycelium advances through a piece of wood. A finite dissociation constant for the haem should not be a problem. The same may be true for a soluble cytochrome *o* in *Rhizobium*, believed to act as an oxygen carrier, and reported to have a periplasmic location [15]. These bacteria grow in nitrogen-fixing nodules in plant roots rather than in liquid suspension. Indeed, the *Rhizobium* provides haem for a plant protein, leghaemoglobin.

Yet there are periplasmic proteins in free-living bacteria that have non-covalent haem. The d_1 haem of cytochrome c_{d1} (nitrite reductase) is not covalently bound [16]. Perhaps such haem groups have unusually low dissociation constants?

Cytochrome b_2 of *E. coli* is stated to have a periplasmic location [6]. The claim that it has homology with cytochrome c' is intriguing [17]; which came first? If protohaem binds to an apocytochrome b_2 (as one might expect), might it also bind to an apocytochrome c', thereby creating a cytochrome b_2?

A further complication is partial export. A soluble cytochrome *o* of *Vitreoscilla*, which acts as an O_2 carrier, was found to be partially exported into the periplasmic space [18]. When cloned in *E. coli*, about 40% was periplasmic [18].

Other prosthetic groups

Haem is not the only prosthetic group that can be covalently or non-covalently bound. The flavin in flavocytochromes *c* from *Chlorobium* and *Chromatium* has a covalent bond to a cysteine side-chain [19]. Another protein with covalently bound flavin and *c*-type haem is *p*-cresolmethylhydroxylase, from *Pseudomonas putida* [20].

Pyrroloquinolinequinone (PQQ) is also a source of analogies. A periplasmic methylamine dehydrogenase contains a closely related derivative of PQQ, covalently bound by cysteine and serine linkages [21]. By contrast, methanol dehydrogenase (also periplasmic) has only non-covalent bonding of PQQ. According to Anthony [21], assembly from apoprotein and free PQQ occurs in the periplasm, and accounts for the substantial amount of PQQ in culture fluids of methylotrophic bacteria.

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