Hypothesis

Why do *c*-type cytochromes exist?

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The hypothesis presented is that the different classes of *c*-type cytochrome originated as proteins located in the bacterial periplasmic space, or on the periplasmic side of the cytoplasmic membrane. In these locations, covalent bonds between haem and protein prevented the haem from being lost to the surrounding medium. Subsequent evolution has led to internal location of *c*-type cytochromes in eucaryotes and cyanobacteria. The covalent links have been retained because of their structural role; a *b*-type cytochrome could be created with similar molecular properties, but its formation would require a large evolutionary jump. If this hypothesis is correct, it should be useful in unravelling electron transport chains with unconventional donors or acceptors. Apparent exceptions deserve further investigation.

Cytochrome c *Haem protein* *Periplasmic protein*

1. INTRODUCTION

Cytochrome *c* contain a haem prosthetic group as in *b*-type cytochromes, haemoglobin or catalase, with the distinction that the haem is attached by covalent bonds, formed by adding cysteine –SH groups across vinyl side chains of protohaem. Among other haem proteins covalently attached haem groups are very rare (e.g. [1,2]). This raises the question of why such bonds should be present in cytochromes *c*.

Ambler [3] has made a start at a classification system for cytochromes *c*. His Class I contains cytochromes showing sequence and structural homology with mitochondrial cytochrome *c*. These include cytochrome c_2 of purple photosynthetic bacteria, soluble *c*-type cytochromes from algal chloroplasts and cyanobacteria (*c*-552, *c*-553, *c*-554), cytochromes c_4 and c_5 from *Azotobacter vinelandii,* and similar cytochromes from a range of other bacteria. The high-spin cytochromes *c'* found mainly in photosynthetic bacteria have a very different structure and are placed in Class II, together with related low-spin cytochromes such as *Agrobacterium tumefaciens* cytochrome *c*-556. Class III contains homologous cytochromes with multiple haems and very low redox potentials, including *Desulfovibrio* cytochrome c_3 and *Desulfuromonas* cytochrome c_7. This leaves a variety of cytochromes *c* for which less sequence and structural information is available. Some are complex proteins with other prosthetic groups in addition to *c*-type haem, for instance the flavocytochromes *c* (nearly always with covalently bound flavin [4,5]), and cytochrome cd_1 in many denitrifiers. Others are intrinsic membrane proteins, in particular cytochrome c_1 of mitochondria and bacteria, and cytochrome *f* of chloroplasts. (Despite its name, cytochrome *f* is a *c*-type cytochrome; it has much in common with cytochrome c_1 [6].) The final number of classes (i.e. unrelated sequences) may run into double figures.

A comparison of *b*- and *c*-type cytochromes provides no evidence that the covalent links confer any novel properties on the haem group. The cytochromes in Class I have a simple electron transferring function; for Classes II and III the function is more debatable (e.g. [7]). Some *c*-type cytochromes bring about oxidation or reduction of an inorganic or organic substrate, in most cases with the aid of

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an additional prosthetic group (e.g. flavin, haem d_1). *Pseudomonas aeruginosa* cytochrome *c* peroxidase is a *c*-type cytochrome with high peroxidative activity [8]. One can find parallels for these roles in proteins with non-covalent protohaem, e.g. cytochromes b_2, b_5 and peroxidase. Redox potentials covering almost the entire biological span are found for both *b*- and *c*-type cytochromes.

2. EVIDENCE AND HYPOTHESIS

Bacterial *c*-type cytochromes have one feature in common: with a few apparent exceptions (discussed below) *c*-type cytochromes are not located in the bacterial cytoplasm. A wide range of soluble *c*-type cytochromes has been shown to be located in the periplasmic zone, or loosely adsorbed on the outer face of the cytoplasmic membrane. These include *Rhodopseudomonas sphaeroides* cytochromes c_2 and c' [9], cytochrome *c*-550 in *Spirillum itersonii* [10], cytochrome c_3 in *Desulfovibrio gigas* and *vulgaris* [11,12], the CO binding cytochrome *c* in *Altermonas haloplanktis* [13], cytochrome cd_1 (nitrite reductase, $NO_2^- \rightarrow N_2O$) in *Ps. aeruginosa* [14], cytochrome *c*-552 (nitrite reductase, $NO_2^- \rightarrow NH_4^+$) in *Escherichia coli* [15,16] and hydroxylamine dehydrogenase in *Nitrosomonas europaea* [17].

There have been fewer reports of the location of soluble proteins with protohaem in bacteria, probably because soluble *b*-type cytochromes are rarely found in energy-conserving electron transport systems. However a cytoplasmic location has been shown for the flavohaemoprotein of *Alcaligenes eutrophus* [18]. It can also be inferred for proteins having NAD(P)H as donor, for instance cytochrome $P\text{-}450_{cam}$ of *Ps. putida,* and other bacterial cytochromes P-450 [19].

Why should *c*-type cytochromes be associated with the periplasm? Any non-covalently bound prosthetic group must have a finite dissociation constant; Granick and Beale [20] cite values up to 10^{-9} M for protohaem. Dissociation of the haem from *b*-type cytochrome is often troublesome during purification. Inside the cell there will be a finite concentration of free haem, so reassociation can occur, as in biosynthesis. But in the periplasm there is a danger that the dissociated haem will diffuse out into the external medium and become lost. Pores in the outer membrane of gram-negative bacteria have an exclusion limit comparable with or slightly above the M_r of free haem (=616) [21].

Apart from the fact that covalent attachment of haem is unnecessary for cytoplasmic proteins, the bond formation requires a specific enzyme, cytochrome *c* synthetase [22,23]. In *Neurospora,* haem attachment occurs as the apoprotein passes through the outer mitochondrial membrane [24]. One of the few bacterial studies has shown the presence of the holocytochrome in the periplasm within a few seconds of protein synthesis [10]. It may be that the location of the bacterial synthetase (which in some cases has to recognise 10 or more haem attachment sites) is such as to prevent reaction with cytoplasmic proteins.

The above examples have all referred to gram-negative bacteria. Comparatively little work has been done on electron transport chains of gram-positive species. However, Jacobs et al. [25] working with *Mycobacterium phlei* found a *c*-type cytochrome with properties typical of Class I. It was located on the outer surface of the cytoplasmic membrane. After removal of the cell wall it could be washed off with 0.15 M KCl. Gram-positive bacteria lack the periplasmic zone of gram-negative ones, and the cell wall can be permeable to hydrophilic substances up to an M_r of 10^5 [26]. Nevertheless, provided ionic or other non-covalent forces are sufficiently strong, extrinsic proteins can still be present on the outer face of the membrane. Thus for the present discussion the distinction between gram-positive and gram-negative is not an important one.

So far the cytochromes considered have been periplasmic soluble proteins, extrinsic membrane proteins, or intermediate between these categories. It is natural to extend the hypothesis to intrinsic membrane proteins. There is good evidence that the haem of mitochrondrial cytochrome c_1 is located near the outer surface of the mitochondrial inner membrane, and the bacterial cytochrome bc_1 complex is very similar to that of mitochondria [6]. In bacteria the same considerations of haem tethering could apply to an outward facing membrane cytochrome as for a periplasmic protein.

Is it true, conversely, that *b*-type cytochromes are absent from the periplasm? This is a less safe assumption. The white-rot fungus *Sporotrichum pulverulentum* produces an extracellular cellobiose oxidase, with spectral properties implying *b*-type haem [27]. The d_1 haem of periplasmic cytochrome

cd_1 is also not covalently bound [28]. Perhaps such haem groups have unusually low dissociation constants? In this respect the claim that cytochrome *b*-562 of *E. coli* has 'vestigial homology' with cytochrome *c'* is intriguing [29]; its location is unclear, according to [30].

Why should eucaryotes contain *c*-type cytochromes, in locations where haem tethering would not seem to be important? This question is also relevant for the *c*-type cytochromes of cyanobacterial thylakoids. As far as is known, these cytochromes are homologous to ones found in other bacteria. They are never located on the same face of the membrane as the proton-translocating ATPase. The majority are either soluble cytochromes in Class I, or membrane cytochromes of the c_1 or *f* varieties, The thioether bonds in *c*-type cytochromes are important in maintaining the correct conformation of the haem group relative to the polypeptide. To dispense with them and still retain the same molecular properties should in principle be possible, but would involve much rebuilding of the protein, and thus is an unlikely evolutionary jump. The result may be the coexistence of soluble *b*- and *c*-type cytochromes in the same compartment, e.g. cytochromes b_2 and *c* in yeast mitochondria [31].

3. COUNTER-EXAMPLES

We must now consider some cases of *c*-type cytochromes reported as being in or adjacent to the bacterial cytoplasm. Trüper and Rogers [32] purified adenylylsulphate reductase from *Thiocapsa roseopersincina*. It was found to have an M_r of 180000 and to contain flavin, non-haem iron and two *c*-type haems per molecule. The enzyme was released by sonication of cells, and must have a cytoplasmic location because one of the natural substrates is AMP. At first sight this seems a clear contradiction of the hypothesis. However, the *c*-type haem has not been shown to be reduced by substrates (as was pointed out in [33]), and the enzyme as purified from several other bacteria contains flavin and non-haem iron, but no *c*-type haem [33]. Further work on this system would be of interest. As a second example (typical of others), Odom and Peck [34] found with *D. gigas* that some haem had a periplasmic location, while rather more was released on lysis of spheroplasts. The cytochromes concerned were not identified. Further work is required to determine whether the cytochromes released before and after lysis are different; if they are identical, then almost certainly some periplasmic material is being retained at the spheroplast stage, for instance because of adhering fragments of outer membrane. The membrane-bound cytochromes *c* of purple sulphur bacteria and a few *Rhodopseudomonads* provide a third type of apparent exception. In these organisms two very different *c*-type haems can be oxidised directly by the photoreactive bacteriochlorophyll, with preferential oxidation of the low-potential haem if both are initially reduced [35]. However, the carotenoid bandshift resulting from flash illumination is higher if the high-potential haem is the one that is photooxidised. This led Dutton and Prince to conclude that the low-potential haem is nearer the cytoplasmic side of the membrane, and drawings in [35,36] show the high-potential haem close to the periplasm (for which there is other evidence), and the low-potential haem adjacent to the cytoplasm. Contrary to such a large separation, magnetic interaction data suggests a spacing of only 8 Å [37]. There is also evidence that both haems are on the same polypeptide [38,39].

Such exceptions are comparatively new in number. Besides, contradictory claims for a protein's location can often be found in the literature (e.g. [14,40]), showing that too much should not be made to hinge on a single unrepeated observation. As things stand, the hypothesis that bacterial *c*-type haem is exclusively found in or near the periplasm certainly holds in almost all cases, and one reason for formulating this rule is to encourage further investigation of apparent exceptions.

4. APPLICATIONS

The chief application is in studies of electron transport chains with unconventional donors or acceptors. The range of inorganic substrates for biological oxidations and reductions is constantly being widened, and bacteria capable of coupling such reactions to energy conservation are usually well endowed with *c*-type cytochromes. Some organic oxidations can also be associated with *c*-type haem, including *p*-cresol, D-gluconate, *N*-methylglutamate and primary amine oxidations [5,41–43]. The distinction between periplasmic

and cytoplasmic compartments is vital for any chemiosmotic model, but evidence of intracellular location is not always easy to obtain. Such evidence becomes unnecessary for *c*-type cytochromes, if *c*-type haem can be assumed to be periplasmic or on the equivalent face of the membrane. Moreover, any model implying direct interaction of *c*-type haem with a soluble cytoplasmic component can be discounted.

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