Biochimica et Biophysica Acta, 1058 (1991) 17–20

ADONIS 000527289100136Z

BBABIO 43395

The functions and synthesis of bacterial *c*-type cytochromes with particular reference to *Paracoccus denitrificans* and *Rhodobacter capsulatus*

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(Received 5 February 1991)

Key words: Cytochrome *c*; Periplasm; Denitrification; Methanol dehydrogenase; Nitric oxide binding

The properties of the *c*-type cytochromes are examined with special reference to their functional value in *Paracoccus denitrificans* and *Rhodobacter capsulatus*.

The diverse modes of growth possible for many genera of bacteria depend critically on the functions of a variety of *c*-type cytochromes [1,2]. This applies particularly to *Paracoccus denitrificans* and *Rhodobacter capsulatus*, and it is these organisms that will be used to illustrate this theme here. It will become clear that for these two well-studied organisms we are some way from understanding the roles of all the *c*-type cytochromes. Some generalisations are possible. For each of these organisms the known *c*-type cytochromes are either periplasmic proteins or membrane-anchored polypeptides with the haem group located at the periplasmic surface of the cytoplasmic membrane. This is in accord with earlier predictions [3,4]. The general occurrence of *c*-type cytochromes in the periplasm of Gram-negative organisms [5] has raised in turn the question of the location and properties of such cytochromes in the Gram-positive bacteria that lack a periplasm [5]. Recently, the amino acid sequence of the cytochrome *c*-550 from *Bacillus subtilis* has been obtained [6]; this is interesting, because a hydrophobic sequence at the N-terminus indicates that the molecule is anchored to the cytoplasmic membrane, in line with previous expectation [5]. This may prove to be a general feature of molecules in Gram-positive organisms where the counterparts in Gram-negative organisms are globular periplasmic proteins; a recently analysed nutrient-binding protein illustrates the same theme [7]. Such anchoring to the cytoplasmic membrane may mean that proteins in Gram-positive organisms function in a similar manner to their truly periplasmic counterparts in Gram-negatives, especially if the periplasm is relatively narrow [8]. However, the absence of many modes of growth (e.g., chemolithotrophy) amongst Gram-positive organisms does suggest that electron transport via water-soluble cytochromes in the periplasm is important [5,9].

Turning to *Pa. denitrificans*, there is evidence from the properties of a mutant that aerobic growth on certain carbon sources, e.g., succinate, is possible in the complete absence of *c*-type cytochromes [10]. However, in the wild type, such aerobic growth is thought to involve part of the electron flow passing through the cytochrome bc_1 complex and cytochrome aa_3 [11]. The connection between the latter two complexes has been thought to involve the periplasmic cytochrome *c*-550 that has sequence similarity with mitochondrial cytochrome *c*. An alternative view, supported by the purification of a supercomplex of cytochrome bc_1 and cytochrome aa_3 with a cytochrome *c*-552, is that the latter is the mediator of electrons [12]. Further evidence for a possible role of cytochrome *c*-552 has recently come from Steinrucke et al. [13] who have purified this protein in detergent and found it to have a mass of approx. 22 kDa. Antibodies raised against it were found to inhibit NADH oxidase activity of membrane vesicles (presumably leaky vesicles, because the catalytic site of NADH dehydrogenase is on the opposite (i.e., cytoplasmic) side of the membrane to the expected location (periplasmic side) of the cytochrome *c*-552) [13]. In contrast, antibodies raised against the cytochrome *c*-550 have been shown in earlier work to be relatively ineffective at inhibiting NADH oxidase activity [14]. It ap-

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pears possible that the main role of cytochrome *c*-550, which has a different distribution of charged residues than mitochondrial cytochrome *c*, may be not to transfer electrons between cytochrome c_1 and cytochrome aa_3 but rather to transfer electrons to and from the periplasm [15]. In this context, it is of interest that there are indications that the amount of cytochrome *c*-550 in cells increases under growth conditions in which periplasmic electron transport is more active [16]. These conditions include growth on C-1 compounds as well as denitrification. Growth of bacteria on methanol or methylamine has been recognised for some time to be dependent on the functioning of *c*-type cytochromes. Recently, the functions of some of these cytochromes in *Pa. denitrificans* has become clearer. Test of methanol dehydrogenase with cytochromes as electron acceptors has shown that only cytochrome *c*-551i (C-L) can act as an acceptor [17]. This finding squares nicely with the finding that inactivation of the gene for cytochrome *c*-551i leads to a loss of of the ability to grow on methanol (Van Spanning, R., personal communication). This cytochrome could be purified in the absence of a detergent, and thus despite the similarity of its molecular weight to the cytochrome *c*-552 discussed above it is probably distinct, especially since experiments with spheroplasts have shown that cytochrome *c*-551i is not directly oxidised by the membrane bound oxidase activity and added cytochrome *c*-550 is needed to mediate the reaction [19]. By default, cytochrome *c*-553, which is synthesised only under conditions of methylotrophic growth, has been suggested as the acceptor from methanol dehydgrogenase, but recent work provides no experimental evidence for this [17]. It has also been suggested that electrons pass from methylamine dehydrogenase via amicyanin to cytochrome *c*-551i, but if this reaction does occur in vivo it is not obligatory, since whereas deletion of a functional gene for cytochrome *c*-551i blocks growth on methanol, it has a much less pronounced effect on growth on methylamine (Van Spanning, R., personal communication). Whilst it appears that cytochrome *c*-551i cannot transfer electrons directly from the periplasm to a membrane-bound oxidase, it cannot be the case that cytochrome *c*-550 is obligatory for this transfer; deletion of cytochrome *c*-550 does not prevent growth on methanol (Van Spanning, R., personal communication).

c-Type cytochromes are also vital for denitrification (reduction of nitrate to dinitrogen gas) by *Pa. denitrificans*. Reduction of nitrate to nitrite does not have this requirement, but all electrons destined for nitrite, nitric oxide or nitrous oxide appear to be routed through the cytochrome bc_1 complex [11]. Cytochrome *c*-550 has been envisaged as the agent for transferring electrons from cytochrome bc_1 to the periplasmic reductases for nitrite and nitrous oxide [11,15]. This view may still prove to be correct, but the finding that cytochrome *c*-550 is dispensable for electron transfer activities of denitrification [20] indicates that one or more other proteins must be able to substitute for it. The nitrite and nitric oxide reductases also contain *c*-type cytochrome [21,22]. In the former, the catalytic site is probably at the *d*-type haem, but studies of the membrane-bound nitric oxide reductase are at such an early stage that it is not possible to suggest whether the *c*-type haem, *b*-type haem or another so-far unidentified redox centre is responsible for the catalytic site.

Pa. denitrificans also contains a number of other *c*-type cytochromes. One of these, subunit molecular mass 45 kDa, has recently been detected in two studies [17,23] and identified as a dihaem cytochrome *c* peroxidase by Pettigrew and colleagues [23]. There are also *c*-type cytochromes in *Pa. denitrificans* that have no known function. A cytochrome c' has recently been indicated [23], plus, at least in some strains, two complexes of *c*-type cytochromes [17]. One of these (150 kDa) comprises a non-haem protein plus four *c*-type cytochrome subunits (28, 33, 41 and 47 kDa). The other is a novel dimeric multi-haem cytochrome *c* (46 kDa) which is made up from subunits of molecular mass 16 kDa and 30 kDa. No function can be suggested for these complexes [17].

At present the electron transfer scheme for *Pa. denitrificans* is essentially as shown in Ref. 11. Cytochrome *c*-553 should probably be deleted from the scheme at present and a cytochrome *c* peroxidase added, accepting electrons from cytochrome *c*-550. It is entirely possible that cytochrome *c*-550 is an electron donor to and from the peripasmic dehydrogenase and reductase systems. The ability of a mutant deleted in cytochrome *c*-550 to transfer electrons to and from these systems [20] may be explained by the appearance of a substitute protein.

In several respects the electron transport proteins of *Rb. capsulatus* resemble those of *Pa. denitrificans*; such similarities include the presence of a cytochrome bc_1 complex and cytochrome c_2, which is similar to cytochrome *c*-550 of *Pa. denitrificans*. Light-driven cyclic electron transport in *Rb. capsulatus* has long been recognised to involve these two components. Specific gene deletion studies have established that cytochrome c_2 is not obligatory for cyclic electron flow [24]. However, the concept that the cytochrome c_1 could pass electrons directly to the reaction centre has recently been challenged by observations that another *c*-type cytochrome can substitute for the absent cytochrome c_2 in the mutant strain [25]. In contrast, cytochrome c_2 is essential in *Rb. capsulatus* for nitrous oxide respiration, one of several anaerobic modes of respiration now recognised for this organism [26]. This has been established by the observation that the cytochrome-c_2-deleted strain was not able to reduce nitrous oxide with electrons derived from physiological substrates yet the reductase

was present [26,27]. This finding makes an interesting contrast with *Pa. denitrificans* in which cytochrome *c*-550 is apparently dispensable for nitrous oxide respiration. Nitrous oxide respiration in *Rb. capsulatus* was originally thought not to involve the cytochrome bc_1 complex [28], but for reasons explained elsewhere it is now clear that electron flow can pass through this complex via cytochrome c_2 to nitrous oxide reductase [29]. Equally clear, however, is the evidence that electrons can reach cytochrome c_2 and thus nitrous oxide reductase via a route that is independent of the bc_1 complex [29]. The cytochrome components of this alternative and myxothiazol-insensitive route may include both *b*- and *c*-type cytochromes, but are not known with certainty. The route is very unlikely to include cytochrome c', which was originally suggested to be a possible electron donor to nitrous oxide in *Rb. capsulatus* [28]. The role of this cytochrome continues to elude definition. Its ability to bind nitric oxide, now generally held to be a free intermediate in denitrification (see Ref. 22 for references), might suggest that it plays a role in maintaining a low concentration of free nitric oxide [30]; the latter is damaging to cells. It has been suggested by R.J.P. Williams [31] that cytochrome c' could be present to signal to cells the presence of nitric oxide and other potentially poisonous molecules. If this is its role it could be that cells will swim away from nitric oxide, implying that cytochrome c' is connected to the chemotaxis system. However, all tested strains of *Rb. capsulatus* are able to reduce nitric oxide [26] which provides an alternative way to remove this potentially toxic molecule.

There is no doubt, however, that nitric oxide can readily bind to many *c*-type cytochromes, including the mitochondrial-type. This has been demonstrated at the low concentrations of nitric oxide that are able to exist in aerobic aqueous solutions [32]. An interesting facet to the reaction of nitric oxide with ferricytochrome *c* is that an electron is donated to the iron of the haem and a species corresponding to a nitrosyl cation is released [32]. Such binding of nitric oxide, followed by release of a derivative of nitric oxide has obvious implications for a signalling system such as that in which nitric oxide is released from endothelial cells [33,34]. In the target cells a guanyl cyclase with a *b*-type haem is believed to be activated by the binding of nitric oxide. By analogy with the reaction of mitochondrial cytochrome *c* with nitric oxide, could the necessary eventual deactivation of the cyclase occur following release of a derivative of nitric oxide formed by gain or loss of an electron?

Whereas cytochrome c_2 is apparently indispensable for nitrous oxide respiration in *Rb. capsulatus*, aerobic respiration can continue in its absence even when an overall pathway involving the bc_1 complex is involved [35]. It has long been known that *Rb. capsulatus* contains many other *c*-type cytochromes other than those mentioned so far, e.g. Ref. 36. The periplasmic nitrate reductase and dimethylsulphoxide reductase each copurify with a specific *c*-type cytochrome. These cytochromes, *c*-556 for dimethylsulphoxide and *c*-552 for nitrate reductase, are probably the direct electron donors to the two reductases in the periplasm, because each is oxidised in the presence of the reductase and appropriate electron acceptor [37–38].

An intriguing question is how the covalent insertion of the haem into *c*-type cytochromes is achieved. In the case of *Pa. denitrificans* it has been shown that the polypeptides for two *c*-type cytochromes, *c*-550 and cd_1, are found in the periplasm of cells in which haem incorporation is blocked either by mutation or inhibition of haem synthesis [21,39]. These findings are consistent with the idea that the polypeptide is translocated independently of the haem to the periplasm, which is where the incorporation occurs. If there are any cytoplasmic *c*-type cytochromes in this organism, as are reported for sulphate-reducing bacteria [40], there would presumably have to be sites of haem insertion in both the cytoplasm and the periplasm. In the case of *Rb. capsulatus*, several types of mutant deficient in *c*-type cytochromes have been described recently [41,42]. There has been difficulty in achieving expression of holo *c*-type cytochromes in *Escherichia coli* [43–44]. This is probably not surprising because *E. coli* does not have many such cytochromes and none is known that resembles the *c*-type cytochrome found in mitochondria to which cytochrome c_2 is related. McEwan et al. [43] found that holocytochrome c_2 from *Rb. capsulatus* was synthesised only in anaerobically grown *E. coli*. It is known that *E. coli* does not synthesise any of its limited repertoire of *c*-type cytochromes under aerobic conditions; such cytochromes are required for reactions involved in anaerobic growth. However, it has recently been shown that the holo cytochrome *c*-550 of *B. subtilis* is expressed in *E. coli* even under aerobic conditions [45]. This has interesting implications; the difference between this result and the observations with the *Rb. capsulatus* gene may reflect that the cytochrome c_2 is a water-soluble protein whereas the *B. subtilis* cytochrome is anchored to the membrane. The conclusion may also be drawn that a cytochrome *c* haem lyase in *E. coli* must be of sufficiently broad specificity to permit incorporation of the haem into heterologous proteins.

In conclusion, although a great deal has been learned about the *c*-type cytochromes of bacteria since the pioneering work of Martin Kamen and others in the 1950's, it is apparent that the diversity of function of this class of molecule means that many aspects of this type of molecule remain to be understood. Perhaps most striking in this regard is the availability of structures for a cytochrome c' and the bound tetrahaem *c*-type cytochrome of the *Rhodopseudomonas viridis* reaction centre. The functions of the two lower-potential

haems in the latter are not known and thus each of these cytochromes provides a rare example of a molecule whose structure is known in the absence of understanding of the function. Whereas there may be many roles for *c*-type cytochromes, we may anticipate that the route of biosynthesis of these molecules in bacteria may prove to be relatively uniform. In this connection it will be important to determine whether there are many examples of *c*-type cytochromes that are exceptional in having cytoplasmic rather than periplasmic locations; the biosynthetic diversity that would ensue from such duality of location is self-evident.

Acknowledgements

This article is based in part on experimental work from the author's own laboratory. That work was supported by SERC and carried out by the colleagues named as co-authors in the references and to whom I am very grateful. Study of *Rb. capsulatus* was a long-standing collaboration with Baz Jackson at the University of Birmingham.

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