Biochimica et Biophysica Acta, 1058 (1991) 52–55

ADONIS 0005272891001281

BBABIO 43404

Two structurally different cytochromes *c* from *Bacillus azotoformans*: on the evolution of Gram-positive bacteria

Gudmundur Oli Hreggvidsson

Institute of Cell and Molecular Biology, Division of Biological Sciences, University of Edinburgh, Edinburgh (U.K.)

(Received 5 February 1991)

Key words: Cytochrome c-552; Cytochrome c-555; Sequence; Evolution; (*B. azotoformans*)

c-552 and split-alpha c-555 cytochromes from *Bacillus azotoformans* are classified on the basis of partial sequence information. The haem-containing polypeptides are postulated to be structurally equivalent to small IC and ID subclass cytochromes found in purple bacteria.

Introduction

Bacillus azotoformans (ATCC 29788 (DSM 1046)) is a Gram-positive soil bacterium capable of denitrifying as well as aerobic respiration [1]. It produces an abundance of *c*-type cytochromes, several of which have been isolated and are currently under study in laboratory. In this paper are presented partial sequences of two *c*-type cytochromes from *B. azotoformans* with distinctly different spectral and structural characteristics; one a *c*-552 cytochrome, the other a cytochrome *c*-555 with a split alpha band [2]. Their structural characteristics and evolutionary significance are discussed in the context of other relevant sequence information from the phylum of Gram-positive bacteria.

The aerobic respiration chain in the genus *Bacillus* is similar in general characteristics to its counterpart in Gram-negative bacteria. Components of the same or similar biochemical and biophysical characteristics are found in both pathways. Thus *c*-type and *b*-type cytochromes as well as *a*- and *o*-type cytochrome oxidases have been reported from *Bacillus* species [3–7]. However, the evolutionary significance of the observed similarities has still to be assessed and this is feasible only on the basis of primary sequence information. Relationships ascertained by the sequencing of electron transport proteins provide a necessary counterpoint to 16S rRNA analysis, which for the Gram-positive phylum has raised as many interesting questions as it has answered.

Cytochromes with spectral characteristics similar to those of the *c*-555 cytochrome from *B. azotoformans* have been reported in low yields from *B. subtilis* [8] and *B. licheniformis* [4,9] grown under conditions of low aeration. Both have a characteristic cytochrome *c*-554 with a split alpha band but so far no sequence information has been available. Cytochromes *c*-551 and *c*-552 from *B. licheniformis* have also been reported [4,9] and the latter has been partially sequenced (Ref. 9; Van Beeumen, personal communication). A complete gene sequence for a *c*-550 cytochrome from *B. subtilis* has also been reported [10]. The only other electron-transfer protein sequence reported from the genus is the *b*-558 cytochrome from *B. subtilis* [2].

Apart from the genus *Bacillus*, *c*-type cytochromes have been reported only from Gram-positive bacteria, to the best of my knowledge, in the aerobic genus *Staphylococcus* [11] which clusters phylogenetically within the *Bacillus* subline in the *Clostridial* (low G + C) subdivision, and in *Mycobacterium phlei* [12] *Microbacterium thermospactum* [13] and *Rhodococcus equi* [14], all of which are aerobic and belong to the *Actionmycetal* (high G + C) subdivision.

Results and Discussion

The *c*-552 and *c*-555 cytochromes from *B. azotoformans* are membrane-bound. They are constitutive and the amount synthesized is approximately the same per unit membrane weight from aerobic and denitrifying cultures. This is in contrast to that reported for the *c*-554 cytochromes from *B. subtilis* and *B. licheniformis*

Correspondence: G.O. Hreggvidsson, Institute of Cell and Molecular Biology, Division of Biological Sciences, University of Edinburgh, Edinburgh, U.K.

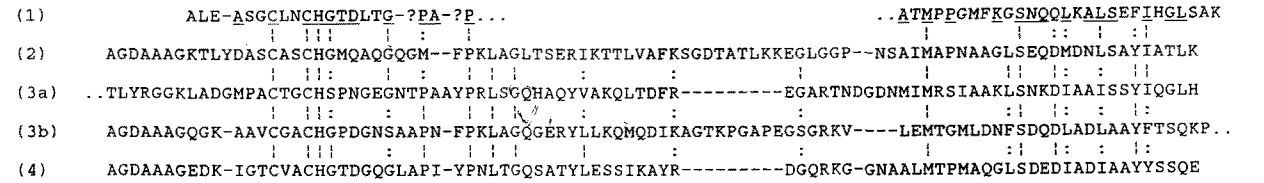

Fig. 1. Alignment of sequence *Bacillus azotoformans* cytochrome *c*-555 with sequences of other bacterial cytochromes *c*. (1) *B. azotoformans* split-alpha cytochrome *c*-555; (2) *Thiobacillus neapolitanus* cytochrome *c*-554(547) [16]; (3a) and (3b) C-terminal and N-terminal halves of *Azotobacter vinelandii* cytochrome c_4 [17]; (4) *Paracoccus halodenitrificans* cytochrome *c*-554(548) [17]. Residues that are identical between sequences are joined by a vertical bar (|) and those that are structurally similar by a colon (:) in what are proposed as signature positions for the class IC sequence class. Residues that are identical in the *B. azotoformans* sequence to any of the other sequences in this figure are underlined.

[3,8,9] which seem to be expressed mainly under conditions of low aeration.

The cytochromes were obtained by tryptic proteolysis from the washed membrane fraction. They were purified by stepwise ammonium sulphate precipitation followed by gel-filtration on Sephadex G-50 and separated by FPLC on a Mono Q column.

Each cytochrome has only two cysteine residues and as deduced from the amino-acid composition the approximate sizes of the cytochromes *c*-552 and *c*-555 are respectively 8700 and 9800. This is consistent with these proteins being small functional haem domains cleaved by trypsin treatment from their N-terminal membrane anchors. Such an anchorage is expected to be a likely feature of cytochromes *c* from Gram-positive bacteria, as they do not have periplasmic space and this has recently been confirmed for *c*-550 from *B. subtilis* by gene sequencing [10].

The haem was removed by treatment with $HgCl_2$ in 0.1 M HCl/8 M urea. The apoproteins were desalted and digested with trypsin. The peptides were separated by G-25 gel filtration followed by high-voltage paper electrophoresis. Peptides were sequenced by standard manual and automated techniques [26].

By aligning the partial sequence of *c*-555 from *B. azotoformans* with sequences of well-characterized cytochromes, a marked similarity was found to cytochromes of the IC subclass [15] (Fig. 1). This subclass includes the split-alpha monohaem cytochromes of *Thiobacillus neapolitus* [16] and *Paracoccus halodenitrificans* [17] and both domains of the dihaem cytochrome c_4, which is found in *Azotobacter vinelandii* [17] and various other purple bacteria of the beta and gamma subgroups. The *B. azotoformans* *c*-555 partial sequence shares approx. 25% identity with comparable regions of each of the set of IC beta purple sequences shown in Fig. 1. However, more notable is the retention of the highly conserved pair of prolines following the haem site and the discernible IC signature, with identical or structurally equivalent residues in key positions.

The *c*-552 cytochrome from *B. azotoformans* and the cytochromes *c*-552 and *c*-550 from *B. licheniformis* and *B. subtilis* are clearly homologous (Fig. 2). A significant similarity is between these cytochromes and the ID subclass (*Pseudomonas* *c*-551 cytochromes) [15]; approx. 30% to each of the set of ID sequences shown in Fig. 2. Also noteworthy is the sixth ligand ID signature Met Pro - - - - - Val and the conserved tryptophan close to the C-terminus. In addition, most of the substitutions amongst the consensus of conserved residues of purple bacteria ID cytochromes are conservative. The most notable difference is a deletion of 11 amino acids which seems to correspond to the region at the distal tip of the loop covering the haem crevice [18]. The preceding region (43–54), shows the greatest divergence to the purple bacteria cytochromes. Interestingly, the tryp-

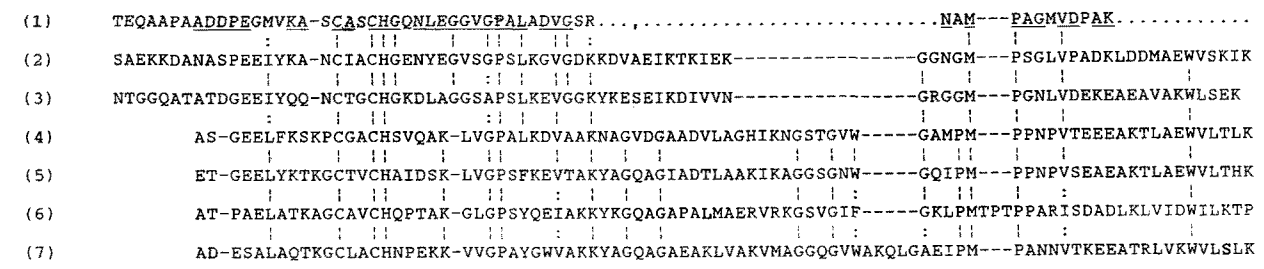

Fig. 2. Alignment of sequence *Bacillus azotoformans* cytochrome *c*-552 with sequences of other bacterial cytochromes *c*. (1) *B. azotoformans* cytochrome *c*-552; (2) *Bacillus subtilis* cytochrome *c*-550 [10]; (3) *Bacillus licheniformans* cytochrome *c*-552 (Ref. 4 and Van Beeumen, J., personal communication; (4) *Pseudomonas mendocina* cytochrome *c*-551 [26]; (5) *Azotobacter vinelandii* cytochrome *c*-551 [27]; (6) *Rhodocyclus tenuis* cytochrome *c*-551 [28]; (7) *Rhodocyclus gelatinosus* cytochrome *c*-551 [28]. Residues that are identical between sequences are joined by vertical bars (|) and those that are structurally similar by a colon (:) in what are proposed as signature positions for the ID sequence. Residues that are identical in the *B. azotoformans* sequence and any of the other sequences in this figure are underlined.

tophan residue −62 is not present. This tryptophan is highly conserved in ID cytochromes and an analogous one is found in different subclasses. The tryptophan is thought to influence the orientation of the haem in the crevice towards the solvent [18] and accords with the observation that the deletion apparently results in the haem being more exposed to the solvent than hitherto encountered in cytochromes *c* of class I.

The occurrence of the IC and ID subclasses in the Gram-positive phylum is unexpected. These subclasses have, so far, been found only in the beta and gamma subgroups of the purple bacteria. Considering the phylogenetic distance between the phyla and the variety of cytochromes *c* of class I, *c*-type cytochromes more specific to the phylum would have been expected.

Oligonucleotide catalogue analysis of 16S rRNA indicates that the ancestral phenotype for gram-positive bacteria was fermentative, similar to the 'true anaerobes' with the deepest branches in the phylum: *Bifidobacteria* of the *Actinomyceatal* division and the clostridia which are found in most sublines of the *Clostridial* subdivision [19]. The occurrence of *c*-type cytochromes in the phylum suggests, however, a much more fully developed respiration mechanism than such an ancestral state would allow for; otherwise, the pathway would be without antecedents in the phylum and would have closer relatives in other phyla. An electron transport pathway of this type and complexity seems either to necessitate a preadapted ancestral system or the acquisition of the cytochromes by lateral gene transfer. In this context it is interesting to note that the species which have been shown to contain cytochromes *c* all belong to phylogenetic clusters which are not particularly deep, which indicates that these groups are not very ancient.

The recent discovery of the Gram-positive photosynthetic species *Heliobacter chlorum* [20], may provide a key to the evolutionary history of the phylum. The available 16S rRNA data for the species [21–23] shows a strong clostridial character and it clusters with *Bacillus* and its relatives [23]. The organism is strictly anaerobic, and cytochromes *c* have been detected on haem-stained SDS gels [20]. On the basis of its discovery, a photosynthetic origin for the Gram-positive bacteria has been proposed [21–23]. However, by the criteria of 16S RNA phylogenetic analysis, it seems that deeper branches must be found to substantiate this claim.

Analysis based on complete sequences of 16S RNA indicates that the Gram-positive bacteria share more recent ancestry with cyanobacteria than with Gram-negative purple bacteria [22]. This does not seem to be supported by the alignment analysis of the *Bacillus* cytochromes above. They have the closest homologues in purple bacteria cytochromes, to the extent of falling into well-defined subclasses from that phylum.

The IC subclass is more closely related to the c_6 subclass (soluble cytochromes *f*) of cyanobacteria than any other, but homology is found only at the C-terminus following the sixth ligand methionine. The c_6 subclass cytochromes show a very high degree of conservation across the great phylogenetic distance between filamentous and unicellular species which have been postulated to have diverged $(1.7–2.0) \cdot 10^9$ years ago [24] and this argues for very ancient sequence characteristics. However, it is possible that the *f* cytochromes may have undergone a major structural change following some fundamental adaption and therefore not retained the N-terminal sequence characteristics seen in the cytochromes from the other two phyla. Therefore, the difference between the Gram-positive and the cyanobacterial cytochromes does not necessarily preclude a special phylogenetic relatedness.

Meyer et al. [25] have proposed that functional and structural restraints limit the number of effective substitutions that can occur in a sequence without drastically altering the physiochemical properties of the protein. Thus, with time there should be increasing incidences of convergent mutations until an equilibrium of dissimilarity is reached. Proteins from a range of distantly related species would then remain sequentially equidistant from one another with fundamental characteristics intact. This interpretation would suggest that genes for both the ID and IC cytochromes were present in the ancestor of both the Gram-positive and -negative bacteria and a conservation of functional mode has preserved these specific solutions to the cytochrome fold. However, the alternative explanation, a more recent vertical transmission of the purple bacteria cytochromes by lateral gene transfer into the ancestral species to the genus *Bacillus* and its close relatives, the genus *Staphylococcus* and *H. chlorum*, must also be considered. Such an event may be indicated by the seemingly sporadic occurrence of cytochromes *c* in species in the top branches of the 16S rRNA tree, the genealogical fermentative substructure of the phylum and resultant difficulties of accommodating the photosynthetic species *H. chlorum*. In theory, it should be possible to find evidence for such an event by affinity analysis of the cytochromes from the different purple bacteria subgroups. However, happening at an early stage in the evolutionary history of Gram-positive bacteria, an equilibrium of dissimilarity may still be manifested and obscure specific relationships.

It may seem that, as more sequence information becomes available, the evolutionary history of the Gram-positive bacteria is becoming more elusive and at this stage there is no want of speculative hypotheses. However, complete sequences of 16S rRNA and electron carrier proteins are sparse and with representatives covering the whole metabolic spectrum of Gram-positive bacteria, possibly some, if not all, of the apparent contradictions will be resolved.

Acknowledgements

I thank Richard P. Ambler for valuable comments and encouragement during this work and Linda Kerr and Steven Peacock of the Welmet Protein Characterization Facility and Margret Daniel for skilled and enthusiastic assistance.

References

1 Pichinoty, F., De Barjac, H., Mandel, M., Greenway, B. and Garcia, J.-L. (1976) Ann. Microbiol. 127B, 351–310.
2 Meyer, T.E. and Kamen, M.D. (1982) Adv. Protein Chem. 35, 105–212.
3 Hederstedt, L. (1986) Methods Enzymol. 126, 399–414.
4 Woolley, K.J. (1987) Arch. Biochem. Biophys. 254, 376–379.
5 Miki, K., Sekuzu, I. and Okunuki, K. (1967) Annu. Rep. Biol. Works Fac. Sci. Osaka Univ. 15, 33–58.
6 De Vrij, W., Azzi, A. and Konings, W.N. (1983) Eur. J. Biochem. 131, 97–103.
7 James, W.S., Gibson, F., Taroni, D. and Poole, R.K. (1989) FEMS Microbiol. Lett. 58, 277–282.
8 Miki, K. and Okunuki, K. (1969) J. Biochem. (Tokyo) 66, 831–854.
9 Woolley, K.J. (1984) Ph.D. Thesis, University of Edingburgh.
10 Von Wachenfeldt, C. and Hederstedt, L. (1990) J. Biol. Chem. 265(23), 13939–13948.
11 Keilin, D. (1980) Proc. R. Soc. London. Ser. B. 98, 312.
12 Jacobs, A.J., Kaira, V.K., Cavari, B. and Brodie, A. (1979) Arch. Biochem. Biophys. 194, 531–541.
13 Davidson, C.M. and Hartree, E.F. (1968) Nature (London) 220, 502–505.
14 Reddy, C.A. and Kao, M. (1982) J. Gen. Microbiol. 128, 2379–2383.
15 Ambler, R.P. (1982) in From Cyclotrons to Cytochromes, pp. 263–280, Academic Press, New York.
16 Ambler, R.P., Meyer, T.E., Trudinger, P.A. and Kamen, M.D. (1985) Biochem. J. 227, 1009–1013.
17 Ambler, R.P., Daniel, M., Melis, K. and Stout, C.D. (1984) Biochem. J. 222, 217–227.
18 Salemme, F.R. (1977) Annu. Rev. Biochem. 46, 299–329.
19 Fox, G.E., Stackebrandt, E., Hespell, R.B., Gibson, J., Maniloff, J., Dyer, T.A., Wolfe, R.S., Balch, W.E., Tanner, R., Magrum, L., Zablen, L.B., Gupta, R., Bonen, L., Lewis, B.J., Stahl, D.A., Luehrsen, K.R., Chen, K.N. and Woese, C.R. (1980) Science 209, 457–463.
20 Fuller, R.C., Sprague, S.G., Gest, H. and Blakenship, R.E. (1985) FEBS Lett. 182(2), 345–349.
21 Woese, R.C., Debrunner-Vossbrinck, B., Oyaizu, H., Stackebrandt, E. and Ludwig, W. (1985) Science 229, 762–765.
22 Woese, C.R. (1987) Microbiol. Rev. 51(2), 221–271.
23 Stackebrandt, E., Pohla, H., Kroppenstedt, R., Hippe, H. and Woese, C.R. (1985) Arch. Microbiol. 143, 270–276.
24 Aitken, A. (1988) Methods Enzymol. 167, 145–154.
25 Meyer, T.E., Cusanovich, M.A. and Kamen, M.D. (1986) Proc. Natl. Acad. Sci. USA 83, 217–220.
26 Ambler, R.P. and Wynn, M. (1973) Biochem. J. 131, 485–494.
27 Ambler, R.P. (1973) Syst. Zool. 22, 554–565.
28 Ambler, R.P., Meyer, T.E. and Kamen, M.D. (1979) Nature (London) 278, 661–662.