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Biochimica et Biophysica Acta 1229 (1995) 303–314

BBA Biochimica et Biophysica Acta

Review

The respiratory chain of alkaliphilic bacteria

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Received 12 January 1995; accepted 18 January 1995

Keywords: Alkaliphile; Cytochrome; Respiration; Oxidative phosphorylation

Contents

1. Introduction

Respiration in the alkaliphilic bacteria is remarkably insensitive to pH; it transduces redox energy at extremely alkaline pH at least as effectively, and perhaps more so, than at near neutral pH [1]. In continuous culture, the facultative alkaliphile *Bacillus firmus* OF4 exhibits rapid growth on nonfermentative substrates up to about pH 11.0, with growth, albeit much slower, observed as high as pH 11.4, the highest pH tested to date [1]. Significantly, the growth yield on the nonfermentable substrate is higher at pH 10.5 than at pH 7.5. The upper growth pH limit for obligate alkaliphile strains may be higher than for facultative strains. Obligate strains differ from facultative strains in that they grow poorly *below* pH 8.5; one such strain, *B. firmus* RAB, outcompetes *B. firmus* OF4 in a chemostat at pH 10.5 [2]. Respiration at pH values in the 10–11.4 range must not only energize the work functions normally associated with bacteria, such as macromolecule biosynthesis and motility, but must also provide the energy for pH homeostasis that is critical to survival at pH values above 9. The maintenance of an internal pH more acidic than the external pH by over 2 units substantially reduces the proton electrochemical gradient available to cells for oxidative phosphorylation, yet this vital process is manifestly robust.

It might be expected, therefore, that these bacteria that thrive at high pH on nonfermentable substrates show

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SSDI 0005-2728(95)00024-0

demonstrable adaptations in their respiratory chain. Other bacteria have been shown to adapt to environmental or developmental changes by alterations in their respiratory chain. For example, *Escherichia coli* normally uses cytochrome *bo* as the predominant terminal oxidase but when the oxygen tension becomes low, *E. coli* synthesizes a different terminal oxidase, cytochrome *bd*, that has a higher affinity for O_2 [3]. *Paracoccus denitrificans* is notable for the considerable flexibility it exhibits in its respiratory chain composition as a function of growth substrate or oxygen tension [4]. High cytochrome levels were first observed in the obligately alkaliphilic bacteria [5]. Subsequently, it was found that facultatively alkaliphilic bacteria have elevated levels of cytochromes when grown at very alkaline pH relative to the levels observed near neutral pH [6,7], indicative of an adaptive response to growth at high pH. Detailed investigations of the various electron carriers in alkaliphile membranes also indicate that the redox midpoint potentials of alkaliphile cytochromes tend to be markedly lower than those of their counterparts in neutralophilic bacteria and mitochondria [8,9].

The alkaliphilic bacteria that we will be concerned with here are eubacterial, Gram-positive, obligately aerobic *Bacillus* species. They have a conventional morphology with no evidence of internal organelles or other unusual vesicular structures associated with growth at high pH [1,10–12]. Electron transfer and ATP synthesis via an F_1F_0 ATP synthase take place on the cytoplasmic membrane and membrane vesicles devoid of cell wall content exhibit the same major bioenergetic properties as those of intact cells [13,14]. A list of the alkaliphilic organisms that have been used in studies related to the respiratory chain is given in Table 1.

This review will focus first on the individual components of the respiratory chain of extreme alkaliphiles and then more briefly note issues regarding how respiration is coupled to energy conservation. We will not discuss the extensive work on a different group of organisms that are alkaline-tolerant [32]. Alkaline-tolerant bacteria thrive near neutral pH and can tolerate pH values up to pH 8.5 or 9.0 and sometimes higher; however, they do not grow optimally above pH 9.0. Some of these organisms are marine bacteria, such as *Vibrio* species, that exhibit primary, respiration-driven, sodium ion translocation [33]. Although Skulachev and colleagues [34] have suggested a wide occurrence of such primary Na^+ pumps, primary sodium translocation has not yet been demonstrated in non-marine, extreme alkaliphiles. Important ancillary issues that arise in the context of alkaliphily will be discussed where the data impinge on the issue. Such issues include pH-mediated regulation of gene expression and functional considerations in connection with segments of membrane proteins exposed to the alkaline *milieu*. For comparative purposes, reference will be made to analogous components of neutralophilic *Bacillus* species, especially those of *B. subtilis* and *Bacillus* PS3, for which the most is known.

2. *B. firmus* OF4 respiratory chain and membrane lipids

2.1. Respiratory chain of *B. firmus* OF4

A speculative model of the respiratory chain of facultatively alkaliphilic *B. firmus* OF4 is presented in Fig. 1. Two types of NADH dehydrogenase, I and II, are inferred to coexist in the energy-coupling membrane (Section 3.1).

Table 1
Alkaliphilic organisms that have been used in studies of respiratory and other heme-containing enzymes

Organism	Facultative or obligate	Respiratory or other heme-containing enzyme	Reference(s)
Bacillus YN-2000	facultative	cytochrome aco_3	[23–25]
		cytochrome *c*-553	[7]
		cytochrome *c*-552	[7]
		catalase	[26]
Bacillus C-125	facultative	gene exhibiting sequence similarity to NADH dehydrogenase	[30,31]
B. firmus OF4	facultative	cytochrome caa_3	[18,19]
		cytochrome *bd*	[20]
		Rieske Fe/S center	[21]
		NADH dehydrogenase	Table 2
		succinate dehydrogenase	Table 2
		catalase isozymes I–III	[22]
Bacillus YN-1	obligate	NADH dehydrogenase	[27–29]
Bacillus alcalophilus	obligate	membrane-bound electron transfer carriers	[5,8]
B. firmus RAB	obligate	membrane-bound electron transfer carriers	[5,9]
		cytochrome caa_3	[15]
		cytochrome *c*-552	[16,17]
		cytochrome *b*-558	[16]

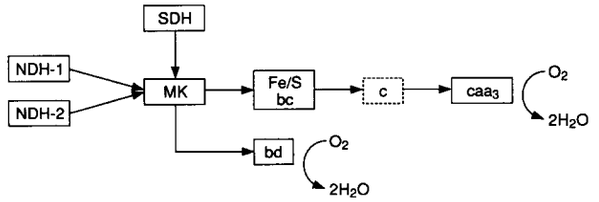

Fig. 1. Hypothetical model of the respiratory chain of *B. firmus* OF4. Abbreviations: NDH-1 and NDH-2, proton-translocating and nonproton-translocating NADH dehydrogenases, respectively; SDH, succinate dehydrogenase; MK, menaquinone; Fe/S and *bc*, cytochrome *bc* complex containing the Rieske Fe/S center; *bd*, cytochrome *bd* terminal oxidase; *c*, cytochrome *c*; caa_3, cytochrome caa_3 terminal oxidase. The dashed lines around cytochrome *c* indicate that it is unknown whether a cytochrome *c* distinct from the cytochrome *c* covalently bound to the caa_3 terminal oxidase is required to mediate electron transfer from the *bc* complex to caa_3.

Consistent with the utilization of the growth substrate, sodium D,L-malate, everted membrane vesicles catalyze succinate dehydrogenase activity. As with other *Bacillus* species, the quinone is menaquinone [9,35]. The presence of a cytochrome *bc* complex had been proposed on the basis of spectral and midpoint potential analyses of membrane redox species [8]. The properties of its Rieske [2Fe-2S] center have been detailed [21]. Two terminal oxidases are expressed under some growth conditions. Cells grown exponentially at pH 7.5 or to stationary phase at pH 7.5 or 10.5 synthesize cytochrome *bd* [20], which functions as a quinol oxidase in other organisms. The other terminal oxidase, cytochrome caa_3, is found under all the growth conditions that have been examined [18,20]. It is not known whether the *bc* complex can pass its electrons directly to the fused cytochrome *c* domain of subunit II of the caa_3 complex or requires a distinct cytochrome *c* to mediate the transfer. A hydrophilic cytochrome *c* has been purified from *B. firmus* RAB, but its function remains to be determined [16].

The effects of growth pH on the bioenergetic properties and expression of particular proteins in *B. firmus* OF4 are routinely determined in cells grown at pH 7.5 or pH 10.5. Interestingly, the levels of NADH dehydrogenase (Table 2), succinate dehydrogenase (Table 2), and F_1F_0-ATP synthase [36] do not vary much with growth pH. *c*-type cytochromes [6] and cytochrome caa_3 [18], on the other hand, are elevated more than 2-fold by growth at pH 10.5. The possible significance of the higher cytochrome caa_3 concentration in relation to the energetics of alkaliphily is discussed briefly in Section 4.2.

2.2. Lipids

Although there is a tendency to focus only on the electron transfer components in the respiratory chain, the lipids in which these components are embedded presumably are intricately involved in the structural arrangement of the components and thus influence their functional interaction. The phospholipid head groups of alkaliphiles are highly negatively charged, with phosphatidylglycerol comprising at least 50% of the phospholipid [37,38]. The cardiolipin content is notably high, with levels up to 25% under some conditions in some species. Phosphatidylethanolamine comprises the bulk of the remaining phospholipid. Small amounts of an unusual phospholipid, bis(monoacylglycero)phosphate are found in most, but not all, alkaliphile membranes [38,39]. The neutral lipids include significant amounts of squalene and C_{40} isoprenoids [38]. Differences are observed in the fatty acid content in the membranes from obligate and facultative strains: two obligate strains contain a high concentration of unsaturated fatty acids, while several facultative strains contain almost none, and the high branched chain fatty acid content of obligate strains is somewhat lower in the facultative alkaliphiles. Liposomes prepared from phospholipids isolated from obligately alkaliphilic *B. firmus* RAB have been compared to those isolated from facultatively alkaliphilic *B. firmus* OF4 with respect to their permeability properties [40] subsequent to preliminary findings suggesting that obligate alkaliphiles lose membrane integrity at near neutral pH values [41,42]. The passive permeability of the

Table 2
NADH and succinate dehydrogenase enzyme activities and measurement of heme O in everted membrane vesicles of *Bacillus firmus* OF4

Assay	Activity or amount in vesicles from cells grown at	
	pH 7.5	pH 10.5
NADH to O_2 [a]	0.09 [b]	0.10
Deamino-NADH to O_2	0.06	0.05
NADH to duroquinone (DQ)	1.48	0.80
Deamino-NADH to DQ	0.19	0.13
Succinate to phenazine methosulfate (PMS)/dichlorophenolindophenol (DPIP) [c]	0.41	0.42
Heme O [d]	–	n.d.

n.d., none detected.
[a] Assayed according to Ref. [44] (22° C).
[b] μmol NADH oxidized or DPIP reduced min^{-1} (mg membrane protein)$^{-1}$.
[c] Assayed according to Ref. [48] (37° C).
[d] Protocol for heme extraction and HPLC purification from Ref. [49]; only membranes from pH-10.5-grown cells have been assayed.

liposomes from the facultative alkaliphile is lower than the obligate alkaliphile at either near neutral or high pH. Valinomycin-mediated $^{86}Rb^+$ efflux is the same at either pH in the facultative preparation but is more rapid near neutral pH than at high pH in the obligate preparation. These results support the hypothesis that the failure of the obligate species to thrive near neutral pH is due to a compromise in membrane integrity [38]. The hypothesis is further supported by the finding that inclusion of unsaturated fatty acid in the growth medium of a facultative strain results in a loss of the capacity of the organism to grow near neutral pH [2]. In the permeability studies, the effects of particular neutral lipid fractions on permeability have also been examined. The inclusion of the isoprenoid fraction in the phospholipid liposomes from either strain lowers the permeability to solutes while the inclusion of the diacylglycerol fraction increases the rate of solute efflux [38].

3. Specific components

3.1. NADH and succinate dehydrogenases

Biochemical and genetic studies in *E. coli* and *Salmonella typhimurium* have demonstrated the presence of two distinct types of NADH dehydrogenase, referred to as NDH-1 and NDH-2 [43,44]. NDH-1 is analogous to the mitochondrial NADH dehydrogenase and is a multisubunit, proton-translocating complex. On the other hand, NDH-1 is composed of a single polypeptide and does not translocate H^+. Mutants lacking NDH-1 activity show several phenotypes, including a decrease in energy-dependent proteolysis that normally occurs after carbon starvation [45]. They also suffer a competitive disadvantage relative to the wild type in stationary phase [46]. The two forms have different substrate specificities, with NDH-2 being unable to utilize reduced nicotinamide hypoxanthine dinucleotide (deamino-NADH or d-NADH) [47]. Thus, at least for these enteric bacteria, the activity of NDH-1 can be determined separately from NDH-2 by using d-NADH as the oxidizable substrate.

Everted membrane vesicles from *B. firmus* OF4 catalyze NADH and d-NADH oxidation with either oxygen or duroquinone (+KCN) as the terminal electron acceptors (Table 2). The results indicate that both NADH and d-NADH are substrates of the respiratory chain, in contrast to *B. subtilis*, which only oxidizes NADH [50]. The results with *B. firmus* OF4 suggest that both NDH-1 and NDH-2 may be present, but since d-NADH is a substrate of the NDH-2 enzyme of *Thermus thermophilus* [51], caution must be taken in interpreting these results.

An NADH dehydrogenase purified from alkaliphilic *Bacillus* YN-1 [27] was determined to be a homodimer with a subunit molecular weight of 65 000. It contains one FAD per subunit and appears to lack non-heme Fe. The biochemical properties are consistent with those of the NDH-2 class. The gene encoding the NADH dehydrogenase has been cloned and sequenced [29]. The gene encodes a protein of about 56 000 and the deduced N-terminal amino acid sequence as well as the C-terminal amino acid sequence agree with the sequence of the protein [28]. Interestingly, the deduced sequence is not very similar to NDH-2 of *E. coli*, showing more similarity to thioredoxin reductase [29]. The NDH-2 protein can be separated by proteolysis into two domains, an N-terminal membrane-binding portion and a C-terminal catalytic domain [28]. Only the catalytic domain exhibits sequence similarity to thioredoxin reductase, a soluble protein [29]. Monospecific antibodies against NDH-2 inhibit about half of the NADH oxidase activity of membranes, suggesting that a second type of NADH dehydrogenase may also be present [29].

More detailed studies of the number and roles of NADH dehydrogenases in extreme alkaliphiles are likely to be of interest in view of the finding that mutants of alkaliphilic *Bacillus* C-125 with partial deficiencies in NADH dehydrogenase exhibit a non-alkaliphilic phenotype [30]. In addition, a gene putatively encoding a Na^+/H^+ antiporter from the same organism recently was sequenced and shown to possess an N-terminal domain with significant sequence homology to NADH dehydrogenase [31].

Substantial succinate dehydrogenase activity is found in *B. firmus* OF4 everted membrane vesicles (Table 2). The *B. subtilis* succinate dehydrogenase complex has been thoroughly studied at the biochemical and genetic level and contains low- and high-potential cytochrome *b* species [52]. Thus, it is likely that the alkaliphile succinate dehydrogenase complex contributes to the multiple *b*-type cytochromes that are resolved in the membranes of alkaliphiles by midpoint potentials (Section 3.4).

3.2. c-type cytochromes

All of the *c*-type cytochromes isolated from alkaliphiles share two distinguishing properties: they are relatively low midpoint potential electron carriers with distinctly low isoelectric points. Fig. 2 illustrates how the midpoint potentials of a number of different *c*-type species of alkaliphiles, including those in the membrane and the isolated forms, compare to a group of those isolated from neutralophiles. The alkaliphile cytochromes are bunched together from about +50 to about +100 mV, while the neutralophilic forms are grouped together from about +180 to +250 mV. Yumoto et al. [7] suggest that the low midpoint potentials of the *c*-type cytochromes may facilitate the transfer of electrons from the outside of the plasma membrane to the a_3 or o_3 moiety on the inside of the membrane that might otherwise be retarded by the large membrane potential, negative inside, found in alkaliphiles at very alkaline growth pH.

Redox titrations of the membrane-bound cytochromes

yield quite low midpoint potentials for the *c*-type, with values of +60 and +70 mV for *B. firmus* RAB and *B. alcalophilus*, respectively [8,9]. These results must represent composite values of multiple *c*-type species in the membranes because multiple heme staining membrane polypeptides are resolved by SDS-polyacrylamide gel electrophoresis (Fig. 3). When no special precautions are taken to retain noncovalently bound heme, this procedure is believed to stain only *c*-type cytochromes due to the covalent attachment of the heme.

The major heme-staining polypeptides of *B. firmus* OF4 have relative molecular weights of 38000, 28000, 16000 and 10000 (Fig. 3). As predicted from the reduced vs. oxidized difference spectra that show that *c*-type cytochromes are up-regulated by growth at high pH, membranes from pH-10.5-grown cells appear to contain higher levels of these polypeptides compared with membranes from pH-7.5-grown cells. The molecular masses of the heme-stained bands of *B. firmus* OF4 are similar to those revealed by biosynthetic 5-amino[^{14}C]levulinic acid labeling of the hemes (a more sensitive method of detection) of the membrane-bound cytochromes of *B. subtilis*, which exhibit values of 52, 36, 29, 22 and 16 kDa [58,59], except that there is no apparent analogue of the 52 kDa polypeptide present in the alkaliphile preparations. At present, only the 36 and 16 kDa polypeptides of *B. subtilis* have been positively identified, as subunit II of cytochrome caa_3 and *c*-550, respectively [60]. Of the alkaliphile heme-staining bands, only the 38 kDa staining polypeptide has been unambiguously identified, as subunit II of cytochrome caa_3.

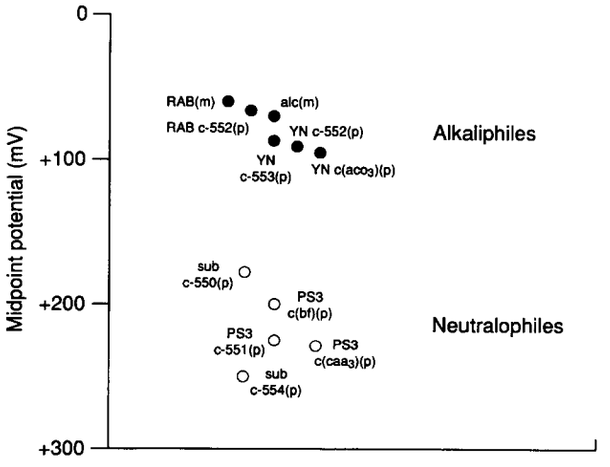

Fig. 2. Midpoint potentials of *c*-type cytochromes of alkaliphiles compared to those of selected neutralophilic *Bacillus* species. The abbreviations are RAB, *B. firmus* RAB; alc, *B. alcalophilus*; YN, *Bacillus* YN-2000; sub, *B. subtilis*; PS3, *Bacillus* PS3; m, membrane (composite midpoint potential of membrane-bound *c*-type cytochromes); p, purified protein. The data are taken from the following references: RAB(m), [9]; alc(m), [8]; RAB *c*-552, [16]; YN *c*-552 and *c*-553, [7]; YN *c*(aco_3), [54]; sub *c*-550, [53]; PS3 *c*(*bf*), [54]; PS3 *c*-551, [55]; PS3 *c*(caa_3), [56]; sub *c*-554, [57].

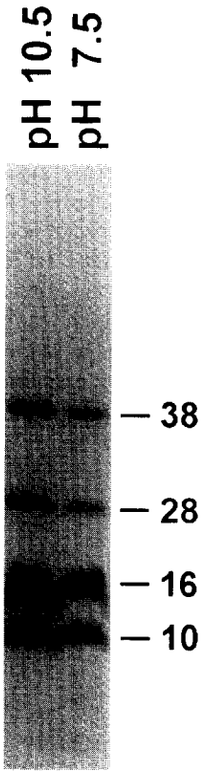

Fig. 3. Heme-stained polypeptides of everted membrane vesicles of *B. firmus* OF4. The molecular mass values of the heme-stained bands are indicated on the right margin, in kDa. The lanes are loaded with 300 μg of membrane protein and are designated by the pH of the growth medium.

A soluble cytochrome *c*, *c*-552, has been isolated from the closely related alkaliphile, *B. firmus* RAB [16]. Consistent with the membrane redox titrations, *c*-552 has a midpoint potential of +66 mV at pH 7 (above pH 8.3, it was pH-dependent). Resonance Raman studies of the purified *c*-552 suggest that its low potential may be due, in part, to a switch in the sixth ligand from methionine to histidine when the oxidized cytochrome is reduced [17]. The mass of the protein by SDS PAGE is 16.5 kDa and it is very acidic, with a p*I* of 3.4 [16]. As isolated, *c*-552 is readily autoxidizable. The function of *c*-552 is not known.

Two membrane-bound *c*-type cytochromes have been purified from alkaliphilic *Bacillus* YN-2000 [7]. Cytochrome *c*-553 consists of a single polypeptide of 10500, with a midpoint potential of +87 mV and an isoelectric point of 3.9 [7]. It is an effective donor to the *Bacillus* YN-2000 terminal oxidase cytochrome aco_3, with a micromolar K_m, but requires the presence of the polycation poly(L-lysine) for activity. The concentration of cytochrome *c*-553 is elevated, as is the aco_3 concentration, in cells grown at high pH. In contrast to cytochrome *c*-553, cytochrome *c*-552 purified from *Bacillus* YN-2000 is not a substrate for the aco_3 and its function remains to be determined. This protein is a multi-subunit complex

with sizes of 40, 32, 19, 17, 14 and 12 kDa, the latter two polypeptides staining for heme. Like *c*-553, the *c*-552 is low potential (+91 mV) and acidic (p*I* of 4.0) [7].

Low-molecular-mass, membrane-bound, *c*-type cytochromes for which the structural genes have been cloned have been purified from two neutralophilic *Bacillus* species. The size of cytochrome *c*-550 from *B. subtilis* is about 13.5 kDa and it is somewhat acidic (with a predicted p*I* of 5.3), and high potential (+178 mV) [53,58]. It is found to be anchored to the membrane by one transmembrane-spanning segment, which can be cleaved off by trypsin, leaving a water-soluble cytochrome with properties indistinguishable from the membrane-bound form [53]. Mutants that are deleted for the gene (*cccA*) grow normally on minimal or rich media and appear to synthesize another *c*-type cytochrome in the absence of the *ccc*A gene product [58]. A 10 kDa cytochrome *c*-551 has been purified from *Bacillus* PS3. It, too, is acidic and high potential [55,61]. Unlike *B. subtilis c*-550, *c*-551 lacks a transmembrane portion and instead appears to be anchored to the membrane by acylation [61].

A careful analysis of the *c*-type cytochromes of *B. subtilis* indicates that all of them are membrane-bound [58], as might be expected for a Gram-positive organism that lacks a true periplasmic space. Probably, the soluble *c*-type cytochromes like the *B. firmus* RAB *c*-552 repre-

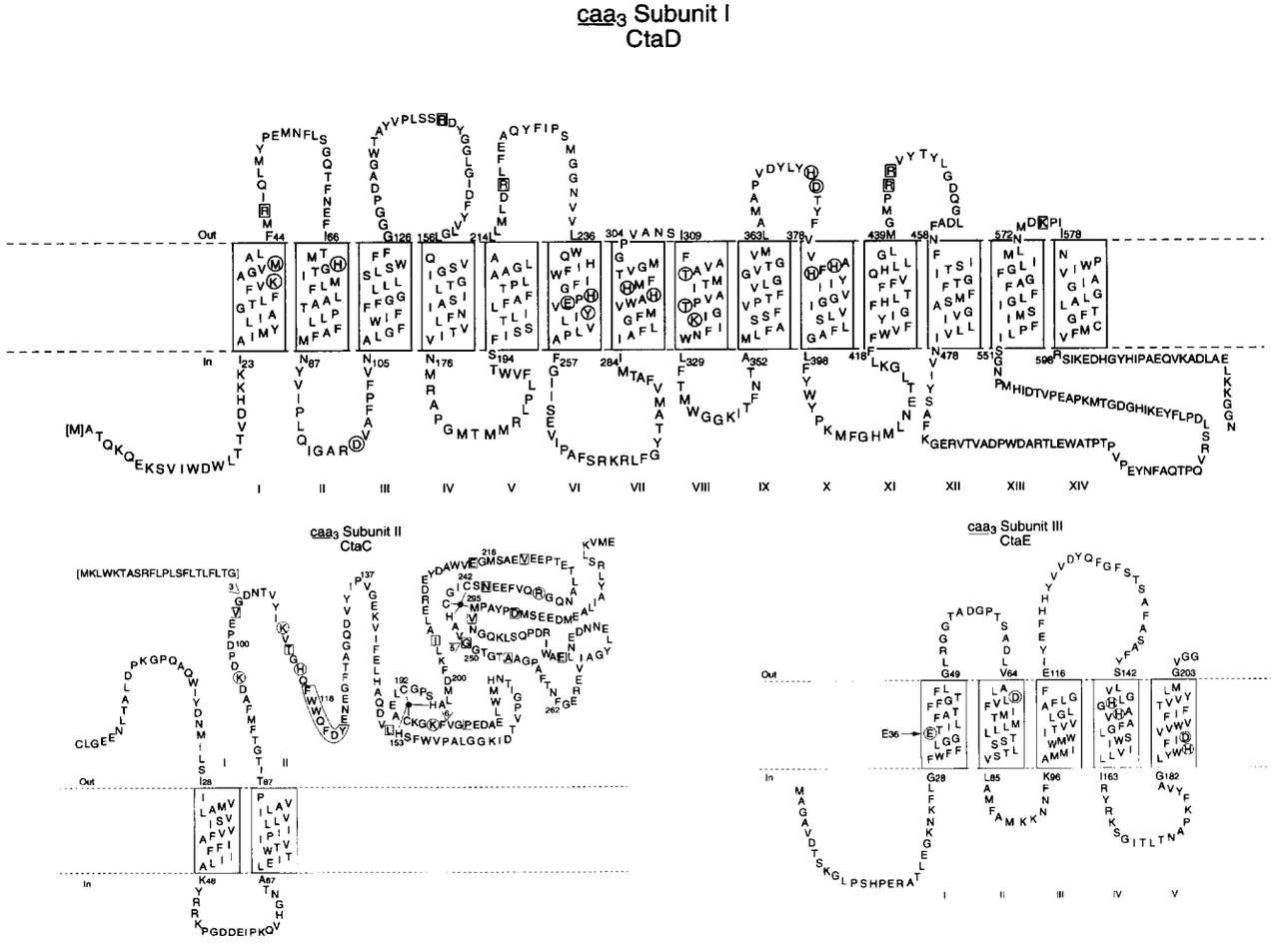

Fig. 4. Topological models for subunits I–III of *B. firmus* OF4 cytochrome caa_3, based on models for cytochrome aa_3 subunits [63,66,68] and for subunit I of cytochrome *bo* [67]. Symbols for subunit I: boxed residues are basic residues that are in putative external loops and circled residues are ones that are proposed to be involved in Cu_B, heme A, and heme A_3 binding or otherwise implicated in function. See text for details. Symbols for subunit II: the bracketed sequence in the upper left is cleaved off in the mature polypeptide; carets indicate gaps in the *B. firmus* OF4 sequence that must be inserted to maintain alignment with the sequence of subunit II of the *B. subtilis* caa_3 [63] and the number after the caret is the number of residues of the gap. Boxes designate residues deviating from the basic residues found at those positions in subunit II from *B. subtilis* and *Bacillus* PS3 [64] and circles show the basic residues that are found only in *B. firmus* OF4. The boxed aromatic-rich set of residues, FWWQFDY, is conserved in *Bacillus* species except, notably, for D122, which is a proline in *B. subtilis* and *Bacillus* PS3. H153, C188, C192, and H196 are putative Cu_A ligands [68], C242, C245, H246 form a consensus CXXCH heme attachment site and M295 is a possible ligand to the heme. The residue numbering is that of the mature protein, with the sequence in brackets showing the cleaved sequence. Symbols for subunit III: circles designate histidine or charged residues that are proposed to have an intramembrane location. E36 is equivalent to the glutamate residue covalently modified by *N,N'*-dicyclohexylcarbodiimide in mitochondrial aa_3 [69].

sent cleaved products of the form that functions in vivo in the membrane. Low-molecular-mass *c*-type cytochromes, as evidenced by heme-staining of appropriate fractions on SDS polyacrylamide gels and by reduced vs. oxidized difference spectra, have been found in both soluble and membrane-bound fractions of *B. firmus* OF4; it will be of some interest to purify these proteins, establish whether the soluble forms represent modified forms of the membrane-bound ones, and determine their function.

Other alkaliphilic *c*-type cytochromes include the fused *c*-type found in subunit II of cytochrome caa_3 (*B. firmus* OF4) or cytochrome aco_3 (*Bacillus* YN-2000) and cytochrome *c* of the *bc* complex. The cytochrome *c* of subunit II of the aco_3 complex is low potential (+95 mV) relative to those of neutralophiles (> +200 mV) [25,62]. Nothing has been published regarding the cytochrome components of the *bc* complex from alkaliphiles.

3.3. Terminal oxidases

The first terminal oxidase to have been isolated from an alkaliphile is a cytochrome caa_3-type complex, from *B. firmus* RAB [15]. The purified complex oxidizes cytochrome *c* from a variety of sources and the activity is optimal at pH 6.0 with added lipid. The complex contains 2 mol of heme *a*, 1 mol of heme *c* and 2 mol of copper per mol enzyme. The complex consists of three polypeptides with masses of 56, 40 and 14 kDa. Although the cytochrome *c* component was attributed to the 14 kDa polypeptide, more recent work on the caa_3 complex from *B. firmus* OF4 would indicate that the 40 kDa polypeptide (subunit II) is the most likely candidate for harboring the cytochrome *c*. The *B. firmus* OF4 caa_3 complex has been purified and the genes for the operon (*ctaA-F*) have been cloned and sequenced [19]. The purified protein contains subunits I–III, with masses of 44, 37.5 and 22.5 kDa, with a possible candidate for subunit IV. The deduced amino acid sequence of subunit II is predicted to contain a cytochrome *c* domain, which was verified by heme staining of the purified complex resolved by SDS polyacrylamide gel electrophoresis [19]. A minor caa_3 peak that is resolved by ion-exchange chromatography [19] subsequently has been shown to be composed of the same subunits I and II as the major peak, but lacks subunit III (Hicks, D.B., unpublished data).

Two genes, *ctaA* and *ctaB*, precede the putative 4 structural genes encoding the subunits of the complex, *ctaC-F* in the *B. firmus* OF4 *cta* operon, the same organization as found in the *B. subtilis cta* operon [63]. Northern blots probed with *ctaD* (encoding subunit I) yield a 4 kb message, a size appropriate for the 4 structural genes but too small to include *ctaB*. The amount of this message in pH-10.5-grown cells is 2–2.5-times that of pH-7.5-grown cells, correlating well with the higher level of spectrally detectable caa_3 in the membranes of pH-10.5-grown cells. A larger message of 5 kb is detected by probing with *ctaB*. A 5 kb message would be sufficient to include *ctaB* through *ctaF* but not *ctaA*, which is predicted to be transcribed in the opposite direction. Presumably, this message is not observed when probed with *ctaD* because it is of minor abundance relative to the 4 kb message which would overlap it; the amount of the larger message is not affected by growth pH.

The deduced amino acid sequence of the genes show substantial similarity to those of the corresponding genes encoding the subunits of the cytochrome caa_3 complex from *B. subtilis* [63] and *Bacillus* PS3 [64], and, to a lesser extent, to the analogous *B. subtilis qox* genes encoding the quinol-oxidizing aa_3 complex [65]. The sequence of the subunits can be aligned with the models for the subunits from *B. subtilis* caa_3 and other aa_3 complexes [63,66] and for subunit I of *E. coli* cytochrome *bo* [67], yielding 14 membrane-spanning helices for subunit I (not 12 as originally published [19]) and 2 and 5 transmembrane segments for subunits II and III, respectively, as shown in Fig. 4.

The likely ligands for heme *a* (H69 and H383), for Cu_B (H296 and H297), and for heme a_3 (H381) [68] are conserved in subunit I, as is H246 which is near Cu_B or a ligand to it [68]. Tyrosine-376, equivalent to Y414 in the *Rhodobacter sphaeroides* aa_3 [70], is conserved; when mutated in *R. sphaeroides*, the enzyme has altered optical properties, though it retains enzymatic activity. Other conserved residues whose importance has been suggested by mutagenesis studies carried out in other systems include D98 (= D135 in cytochrome *bo*, a residue implicated in H^+ pumping activity [71]) and the polar residues predicted to be on one face of helix VIII, T311, T318 and K321.

The N-terminal amino acid sequence of subunits I and II show that the N-terminal methionine residue of subunit I is cleaved off and that a more extensive processing of subunit II occurs, with the mature form beginning at cysteine 22. A similar cleavage of subunits I and II of the complex from *Bacillus* PS3 has been demonstrated [64]. Interestingly, there is a consensus sequence around the cysteine of the *Bacillus* subunits that corresponds to that of Gram-negative lipoprotein precursors [65]. However, the *B. firmus* OF4 sequence fits the consensus before the cysteine but not after.

The numbering of the residues of subunit II in Fig. 4 reflects the mature form of the protein. The cleaved sequence is shown in brackets on the upper left of the figure. The carets show gaps of the indicated number of residues that must be used to align the sequence with subunit II of *B. subtilis* [63]. Conserved residues include an aromatic stretch of amino acids (boxed residues 117–123) as well as the residues likely to be involved in ligating Cu_A (i.e., H153, C188, C192 and H196) and those used for C-heme binding (consensus sequence CXXCH; C242-H246). A potential ligand to the heme, M295, is also conserved. Four acidic residues that have been suggested to function

in cytochrome *c* binding, D122, D150, D187 and E212, are conserved as well [66].

A very interesting topological feature of this model is that a number of conserved basic residues predicted to be on the outside are substituted in the alkaliphile protein by neutral or acidic residues (boxed residues, e.g., V103, L152, P181, E216, N250, V267, E280). Consistent with this finding, the cytochrome *c* domain of subunit II has a predicted p*I* of 4.0 compared with 8.0 or above for those of *B. subtilis* and *Bacillus* PS3. The inference is that loops of proteins exposed to the outer surface are stabilized in the alkaline *milieu* by keeping the number of basic residues to a minimum. A similar pattern was found in the *sec*Y gene from a different alkaliphile [72]. As more alkaliphile genes encoding membrane proteins are sequenced, the generality of the inference will become clearer. Such an exaggeration of the usual, "positive in" rule [73] could be a valuable aid in predicting the membrane topology of polytopic alkaliphile proteins. The putative adaptation of minimizing the number of basic residues exposed to the bulk is found in extracellular enzymes of alkaliphiles [74]. Among homologous neutralophilic and alkaliphilic proteases that are secreted, there is a significantly lower content of basic residues in the alkaliphile examples. That "external" domains of membrane proteins exhibited the same adaptation indicates that they in fact experience a similarly high pH near their surface location. In the model of Fig. 4, for example, residue 103 (V103) would be predicted to be less than 50 Å from the membrane surface and its replacement of a conserved basic residue may suggest that V103 is in a more alkaline environment than its homologue in neutralophiles.

The model of subunit III suggests 5 transmembrane helices (Fig. 4). It has been pointed out that those oxidases that have an extended C-terminus on subunit I, creating 2 additional membrane-spanning helices, also have a truncated form of subunit III, lacking two N-terminal transmembrane segments [63,68]. Such is deduced to be the case for *B. firmus* OF4 caa_3. The glutamate residue at position 36 is equivalent to the *N,N*-dicyclohexylcarbodiimide-binding glutamate residue found in subunit III of mitochondrial aa_3 [69].

A cytochrome *a*-containing terminal oxidase has been purified from *Bacillus* YN-2000. Rather than an aa_3-type oxidase, it is an *aco* oxidase with a stoichiometric heme composition of heme A, heme C and heme O of 1:1:1, based on pyridine hemochrome spectra [23,25]. The complex contains about 2 g atoms of copper, 2.5 g atoms of iron, and 1.8 g atoms of magnesium per mole of heme A. Stopped flow and rapid scan measurements of the turnover of the enzyme indicate that heme O reacts with O_2 [24]. Electron flow is suggested to be from heme C to heme O to oxygen [24,25]; the role of heme A in intramolecular electron transfer and its possible relationship to the heme A of mitochrondrial cytochrome aa_3 remains to be elucidated. The complex consists of three polypeptides of 50, 41 (with a bound cytochrome *c*) and 22 kDa. Optical, EPR, and resonance Raman spectra have been used to demonstrate that the complex contains Cu_A [25]. Resonance Raman spectra also indicates that the environment around heme O_3 is similar to that observed with heme A_3 in mitochondrial aa_3 [25]. These data suggest a high degree of similarity between the cytochrome aa_3 family and the *Bacillus* YN-2000 complex, which the authors have designated cytochrome aco_3. The aco_3 complex oxidizes the *Bacillus* YN-2000 cytochrome *c*-553 in the presence of low concentrations of poly(L-lysine) as well as mitochondrial cytochrome *c* in the absence of the polycation [25]. Cyanide inhibits 50% of the activity at a concentration of 7.6 μM [23].

B. firmus OF4 also expresses a type of terminal oxidase, cytochrome *bd* [20], that does not contain heme A, whereas no evidence has yet been presented, to our knowledge, for a second terminal oxidase in *Bacillus* YN-2000. Among *Bacillus* species, *B. subtilis* [75] and *B. cereus* [76] previously had been shown to possess *bd* type oxidases. The expression of the *bd* complex in *B. firmus* OF4 is both growth pH- and growth stage-dependent and differs markedly from the pattern of caa_3 expression [20]. In exponentially-grown cells, *bd* is synthesized only at pH 7.5; in stationary cells, substantial levels of *bd* are observed at both pH 7.5 and 10.5. The lack of detectable *bd* in pH 10.5 exponentially-grown cells indicates that it is not involved in alkaliphily.

In membranes, cytochrome *bd* is reducible by NADH but not by ascorbate + TMPD [20]. The reduced-CO minus reduced spectrum shows that the *bd* reacts with CO, suggesting that it functions as a terminal oxidase. To obtain more information on the complex, it has been solublized with octyl glucoside and partially purified by anion-exchange chromatography. Monospecific antibodies against the *E. coli bd* subunits I and II react in Western blots both with the partially purified preparation and with the corresponding bands in membranes that contain cytochrome *bd* [20].

A *b*-type cytochrome that also has been extracted from membranes and partially purified [20] yields a CO-reduced minus reduced difference spectrum similar to that observed in cytochrome *bo* preparations [77]. However, other oxygen-binding proteins, such as the *Vitreoscilla* hemoglobin-like protein [78] and a *b*-type cytochrome (*b*-558) from *B. subtilis* [79] can be confused with cytochrome *bo* in a static CO spectrum. Heme extraction and HPLC separation of different heme types, the method used to characterize heme O from purified *E. coli bo* [49], has been employed as another approach to assay for the presence of cytochrome *bo* in *B. firmus* OF4 membranes. No heme O is detected in the alkaliphile membranes, although heme O is readily obtained by a corresponding extraction of *E. coli* cells overexpressing cytochrome *bo* (Table 2). These data make it unlikely that cytochrome *bo* is present in exponentially grown *B. firmus* OF4 in significant quantities. More-

over, genes apparently encoding alkaliphile hemoglobin-like protein(s) have recently been cloned [80]. Their product is being characterized and is a good candidate for the ostensible "cytochrome *bo*" species observed in earlier spectral studies.

In the light of very recent findings that at least some bacteria contain numerous terminal oxidases, e.g., *Azorhizobium caulinodans* [81] and *Paracoccus denitrificans* [82,83], some of which can only be detected in mutant strains lacking one or more of the respiratory complexes, further studies of *B. firmus* OF4, especially utilizing genetic disruptions of terminal oxidase genes, will be required to determine whether its terminal oxidase complement is restricted to cytochrome caa_3 and cytochrome *bd*. The data to date are consistent with the conclusion that malate-grown *B. firmus* OF4 utilize one cytochrome *c* oxidase when growing exponentially at high pH. Similarly, *Bacillus* YN-2000 expresses one terminal oxidase that also contains cytochrome *a*. In addition to the need for gene disruptions to reveal the presence of other oxidases, it will also be important to assay quinol oxidase activity in membrane fractions and detergent extracts, which has not been carried out in *B. firmus* OF4 or reported for *Bacillus* YN-2000. Furthermore, no studies have been carried out on the terminal oxidase content of glucose-grown *B. firmus* OF4 or of sporulating forms of alkaliphiles and consequently it is unknown whether the quinol-oxidizing aa_3 found in glucose-grown *B. subtilis* [84] or the two forms of cytochrome aa_3 that have been found in sporulating *B. cereus* [85] also exist in any alkaliphiles.

3.4. b-type cytochromes, including the bc complex, and other heme proteins of alkaliphiles

Redox titrations of membrane vesicles from *B. alcalophilus* and *B. firmus* RAB resolve four *b*-type species with a range of midpoint potentials [8,9]. *B. firmus* RAB *b*-type cytochromes, for example, exhibit potentials of +20, −120, −180 and −400 mV [9]. No membrane-bound *b*-type cytochromes of the alkaliphiles have been purified to homogeneity. Several cytochrome complexes inferred to be present in *B. firmus* OF4 would be expected to contain *b*-type cytochromes, including cytochrome *bd*, succinate dehydrogenase, and the cytochrome *bc* complex, the latter two complexes having high- and low-potential *b*-hemes [52,86].

A soluble *b*-type cytochrome with an α absorption peak at 558 nm has been purified from *B. firmus* RAB [16]. The molecular mass of the protein is about 15.5 kDa and it is very acidic, with an isoelectric point of about 3.1. Unlike cytochrome *c*-552 from *B. firmus* RAB, the midpoint potential of cytochrome *b*-558, which was +28 mV, is pH-independent. The cellular location and the function of this cytochrome have not been determined. It is unlikely to be externally-oriented, like the *c*-type cytochromes, since it would then violate the general rule that only cytochromes with covalently attached hemes are exposed to the external milieu [87].

As mentioned in a previous section, electron transport chains of *Bacillus* species utilize menaquinone, which has a midpoint potential 150 mV lower than ubiquinone [88,89]. Midpoint potentials (determined by EPR) of the Rieske Fe/S centers of *Bacillus* PS3 [89] and *B. firmus* OF4 [21] as well as other Gram-positive organisms are significantly lower than the centers involved in ubiquinone redox chemistry, leading Riedel et al. to propose that the midpoint potentials of Rieske Fe/S centers are largely determined by the corresponding midpoint potential of the associated quinone [21]. The midpoint potential of the alkaliphile center is even somewhat lower than centers from other Gram-positive organisms, with a potential of +105 mV at pH 7 that becomes pH-dependent at pH 8 and above [19]. Apart from its low midpoint potential, the *B. firmus* OF4 Rieske Fe/S center shares additional properties with other menaquinol-utilizing centers that separate them from those involved in ubiquinol or plastiquinol oxidation. These include the effects of particular inhibitors of *bc* complexes, such as DBMIB, on the spectral properties of the EPR signal, and the lack of a redox effect induced by stigmatellin [21].

Another class of heme proteins in alkaliphiles includes catalases, enzymes that degrade hydrogen peroxide. Due to the susceptibility of biological molecules such as menaquinone [84] and ascorbate [90] to autoxidation at alkaline pH values, the alkaliphiles may produce higher concentrations of hydrogen peroxide than neutralophiles, necessitating higher levels of catalase. In fact, the catalase activity of pH-10.5-grown cells is about twice that of pH-7.5-grown cells of *B. firmus* OF4. Three different catalase isozymes in cell extracts have been identified on enzyme-stained native polyacrylamide gels; the isozymes are designated I–III in order of decreasing electrophoretic mobility [22]. These isozymes have been purified and the N-terminal amino acid sequence of each determined [22]. The protoheme-containing isozymes, I and II, are expressed in exponential cells. Isozyme II is responsible for the higher catalase activity at pH 10.5 and is induced to high levels in cells treated with H_2O_2 or ascorbate. Isozyme III, whose spectral properties are similar to other chlorin-containing catalases, is expressed only in stationary phase cells. Interestingly, the two putative chlorin proteins of *B. firmus* OF4, cytochrome *bd* and catalase isozyme III, are found mainly in the stationary phase.

Isozyme III is the product of a gene for which a partial clone has been obtained and sequenced [91]. The gene is 67% identical in the first 449 amino acids to the chlorin-containing HP-II catalase of *E. coli* [92]. Isozyme II is related to the *B. subtilis* catalase *katA* gene product, which was shown to protect cells against an H_2O_2 challenge [93]. Finally, isozyme I exhibited relatedness to a catalase purified from *Bacillus* YN-2000 [26]. Isozyme I and the *Bacillus* YN-2000 enzyme shared some biochemical prop-

erties as well, such as peroxidatic activity with substrates such as guiaicol and the ability to be reduced by dithionite. On the basis of heme spectra of cytoplasmic fractions of *Bacillus* YN-2000, its catalase content is increased in cells grown at high pH relative to neutral pH [26], as is also seen for *B. firmus* OF4 according to enzyme activity measurements [22].

4. Bioenergetics

4.1. Ion translocating specificity of the respiratory chain

There is no evidence in the alkaliphiles for primary Na^+ pumping activity associated with the respiratory chain [32,94–97] and the F_1F_0 ATP synthase does not use Na^+ [98,99]. Sodium ions do not stimulate NADH oxidation [6] or respiration [6,18] in any wild-type alkaliphiles. Proton translocation has been demonstrated concomitant with respiration, and the respiratory chain extrudes H^+ with a high H^+/O ratio [96,97]. By contrast, all Na^+ efflux in alkaliphilic *B. firmus* strains is protonophore-sensitive and hence secondary to primary proton-pumping [94]. Attempts are underway in our laboratory to apply various types of selective pressure to ascertain whether alkaliphile mutants that exhibit primary Na^+ pumping can be isolated. This may not be possible if there are cogent reasons why extreme alkaliphiles lack primary Na^+ pumps. The reasons for the absence of a primary Na^+ cycle in alkaliphiles, or conversely, why alkaline-tolerant species possessing a primary Na^+ pump do not grow at extremely alkaline pH might relate to the finding that the capacity for pH homeostasis was the limiting factor, even for the extreme alkaliphiles at the alkaline edge of their pH range [1]. Perhaps the presence of primary Na^+ pumps in extreme alkaliphiles would interfere with the secondary sodium cycle on which pH homeostasis is dependent. Or, the secondary cycle involved in pH homeostasis may be inadequate in the alkaline-tolerant marine bacteria. Non-alkaliphilic bacteria with primary Na^+ pumps may generate higher $\Delta\Psi$ (transmembrane electrical potential) values than those neutralophiles or alkaline-tolerant bacteria that lack them. However, they still may not be able to regulate their cytoplasmic pH well enough to grow optimally at the more elevated pH values experienced by true alkaliphiles because they lack sufficient activities of the other crucial components of the secondary cycle, involved in pH homeostasis, e.g., sufficient antiporter activity or Na^+ re-entry routes [4,100].

4.2. Coupling of respiration to ATP synthesis

The mechanism by which respiration energizes oxidative phosphorylation in cells growing at high pH is of considerable interest because the bioenergetic patterns do not appear to conform to a strictly chemiosmotic model as originally formulated by Mitchell [101]. The dimensions of the bioenergetic dilemma have been made strikingly clear by the bioenergetic study of *B. firmus* OF4 grown in continuous culture at different pH values [1]. As the pH is increased in increments from 7.5 to 11.2, the protonmotive force (Δp, in mV) declines and then remains constant at a minimal value. Over the same pH range, the phosphorylation potential (ΔG_p) increases to a small degree. As a result, the $\Delta G_p/\Delta p$ ratio increases dramatically as the growth pH is raised, from 3 to 13 [1], with values of 3–4 being typical for other bioenergetic systems [102]. In the chemiosmotic formulation, the $\Delta G_p/\Delta p = H^+/ATP$; the increasing ratio could be accommodated by a strictly chemiosmotic model *only* if the ATP synthase has a remarkably variable H^+/ATP stoichiometry. Perhaps errors in determinations exaggerate the apparent discordance and a small change in stoichiometry would suffice, but this is unlikely. When large artificial diffusion potentials are imposed across cells or ADP + P_i-loaded vesicles at pH values above 9, no ATP synthesis is observed [14,103], i.e., the capacity for ATP synthesis at pH > 9 depends on a natural, respiration-generated primary force. If the F_1F_0 were able to use a variable stoichiometry, one would expect to see substantial ATP synthesis upon imposition of an artificial potential. Moreover, if ever greater H^+/ATP were used at increasing pH, the H^+/O would have to rise even more, since the molar growth yields on malate are greater at pH 10.5 than at pH 7.5 [1]. Thus, whatever the mechanism of oxidative phosphorylation, it is apparently highly energy-conserving, especially at the pH values for growth, rather than the converse [104].

Several lines of preliminary data suggest a special role for the caa_3 of *B. firmus* OF4 in oxidative phosphorylation that may be independent of its role in generating a bulk Δp. There is a correlation between the capacity for oxidative phosphorylation at pH > 9 and the presence of a substantial caa_3 content in the membrane [103]. At the protein level, pH-10.5-grown cells have a 2- to 3-fold higher concentration of caa_3 complex than pH-7.5-grown cells, representing an approx. 4-fold and 2-fold molar excess over the F_1F_0-ATP synthase content, respectively [18,36]. Northern blots indicate that the level of *cta* transcript is elevated to the same extent as is the complex in the membrane [19]. Most likely, the transcriptional regulation is mediated by the low bulk Δp rather than by high external pH, because cells treated with sublethal concentrations of the protonophore carbonylcyanide *m*-chlorophenylhydrazone exhibit a similar elevation in caa_3 complex and the *cta* transcript [18,19]. The Δp-sensing device(s) will be of considerable interest, as will the issue of whether there are also distinct pH sensors associated with the *cta* operon or its products. Genetic and biochemical attempts at dissection of a special direct role, e.g., in a direct transfer of protons to the ATP synthase, will also clarify the structure and function of this important, Δp-regulated complex.

Acknowledgements

This work was supported by a grant from the Department of Energy (DE-FGO2-86ER13559).

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