Association of Chemotaxis with Reduction of Cytochrome *b* in *Bacillus subtilis*

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Addition of chemotactic attractant to *Bacillus subtilis* brought about a transient increase of absorption at 557 nm, compared with absorption at either 543 or 575.5 nm. The increase was tentatively attributed to reduction of cytochrome *b*. This reduction was linked to the ability of attractants (and certain other reagents) to make all bacteria in a population swim smoothly, rather than sometimes swimming and sometimes tumbling as they normally do. It is thought to signify a higher energy requirement for swimming since, in tumbling, flagella may tangle and jam, resulting in periods of no energy loss. Cations were required for motility; the function of the cations was probably not to energize motility, since protons alone could do that, but rather to reduce the surface potential of cells and, thus, avoid excess local acidity.

INTRODUCTION

Chemotaxis is the process by which organisms travel to higher concentrations of attractant or lower concentrations of repellent. Peritrichous bacteria alternately swim smoothly (due to counter-clockwise rotation of flagella, viewed from the flagellum looking towards the cell body; Larsen *et al.*, 1974) and tumble (due to clockwise rotation of flagella; Larsen *et al.*, 1974); the effect of tumbling is to randomly reorientate the bacterium for the next swim. Selective modulation of this 'random walk' causes net migration in the favourable direction: for example, bacteria which happen to be swimming towards higher concentrations of attractant swim longer before tumbling than they would in isotropic medium (Berg & Brown, 1972). In fact, addition of attractant to a suspension of bacteria brings about transient swimming by all bacteria; addition of repellent brings about transient tumbling (Macnab & Koshland, 1972; Tsang *et al.*, 1973; Ordal & Goldman, 1975).

The physiological events that underlie such transitory behavioural change are of great interest. Addition of attractant causes proteins to become methylated (Kort *et al.*, 1975; Springer *et al.*, 1977; Goy *et al.*, 1977) and may affect the distribution of bound and free Ca^{2+} inside the cell (Ordal & Fields, 1977). Addition of repellent to *Bacillus subtilis* may transiently depolarize the membrane (Ordal & Goldman, 1975; Miller & Koshland, 1977) and allow inflow of Ca^{2+} (Ordal, 1977; Ordal & Fields, 1977), which is normally removed from cells by active transport (Rosen & McClees, 1974). In order to explore other possible changes in physiology, I have attempted to observe redox changes in cytochrome *b* as a consequence of addition of attractant.

METHODS

Bacteria. *Bacillus subtilis* strain 01400 was derived from 018, whose lineage is traceable to Marburg 168 (Ordal & Goldman, 1975), by selection for resistance to the proline analogue, azetidine-2-carboxylic acid [see Ordal *et al.* (1978) for details of isolation procedure]. This strain has wild-type $V_{\max}$ for proline transport, but has a K_m of 90 μM , compared with the wild-type value of 2.3 μM (Ordal *et al.*, 1978). It should be noted that the K_t for azetidine-2-carboxylic acid in the wild type is 23 μM for transport and 2.3 μM for chemotaxis, transport and chemotaxis being mediated by different proteins (Ordal *et al.*, 1978).

Strain 01401 was derived from strain 01400 by mutagenesis with ethyl methanesulphonate using the method of Ordal (1976*b*) followed by screening on 'miniswarm' plates (Ordal & Adler, 1974) of 0.55 % (w/v) agar, 1 % (w/v) tryptone, 0.5 % (w/v) NaCl for small dense clones. Clones were grown in tryptone broth and examined microscopically for lack of motility. Strain 01401 was later stained by the method of Leifson (1951) and found to have straight flagella (see below). The strain was sensitive to the flagellotropic phage PBS1, which requires rotating flagella (Martinez *et al.*, 1968).

Strains sc3 and sc4 (Martinez *et al.*, 1968) are mutants of a strain also derived from Marburg 168 and also have straight flagella. Thus, the flagella cannot form a bundle in the back of the bacterium so that it is non-motile. However, the flagella rotate, as do wild-type flagella.

Media, including buffers. Tryptone broth contained 1 % (w/v) tryptone and 0.5 % (w/v) NaCl. L broth contained 1 % (w/v) tryptone, 0.5 % (w/v) yeast extract and 0.5 % (w/v) NaCl. Buffer used in cytochrome absorption experiments contained 1 mM-piperazine-*N,N'*-bis(2-ethanesulphonate) (PIPES), 0.1 mM-EDTA, 0.1 mM-sodium lactate and 56.3 mM-KSCN; the buffer was used at 80 or 100 % strength. Sometimes the EDTA concentration was decreased to 1 or 10 μM (for the experiments on cationic requirements for motility and cytochrome reduction) or the KSCN was replaced by NaSCN at the same concentration (for valinomycin experiments). The pH was about 6.9 to 7.0 in all experiments, unless otherwise noted.

Growth of bacteria. Tryptone broth (1 ml) was inoculated from a frozen culture in 0.5 % (w/v) tryptone, 0.25 % (w/v) NaCl, 10 % (v/v) glycerol and grown overnight; then 0.1 ml was added to 5 ml L broth and two or three serial 25-fold dilutions were made. The cultures were grown with shaking to between 270 (1.2×10^9 bacteria ml^{-1}) and 320 Klett units (filter 66). When bacterial densities were in this range, 2×10^8 bacteria were taken out, filtered and washed with buffer. They were immediately suspended in buffer, sometimes in the presence of specified chemicals, and placed in a 1 ml cuvette at about 1.6×10^8 bacteria ml^{-1} for spectrophotometric observation.

Spectrophotometric measurements. The Aminco-Chance DW2 spectrophotometer was used at room temperature (23 °C) in the dual-wavelength mode for measurements of cytochrome reduction. In this mode, monochromatic light of two different wavelengths was sent through the sample on a time-shared basis at 250 cycles per second, and the signal indicated the difference between the absorptions at the two wavelengths. For measurements of cytochrome *b* reduction, $A_{557} - A_{575.5}$ was normally used. The scale of 0.01 absorbance unit full scale was used. Samples, usually 1 μl but occasionally up to 3 μl , were added using a thin glass rod and the suspension was rapidly stirred. The response was compared to that from previous stirrings where no reagent was added.

RESULTS

Effect of addition of attractant on cytochrome absorption. Experiments were carried out to test whether any change of absorption of cytochrome *b*, as a representative of the electron transport chain, occurred as a consequence of the addition of attractant. To minimize any change in attractant concentration following addition, the strain chosen for these studies was a mutant resistant to azetidine-2-carboxylic acid (a proline analogue with a four- rather than a five-membered ring): its K_m for proline is 40 times higher than that of the wild type (see Methods). Transport of the analogue in this strain is extremely sluggish, the concentration being calculated to decrease from 7.3 to 7.29 μM in 15 s, the duration of the experiment.

Cytochrome *b* has an absorption maximum for the reduced form at 557 nm and local troughs at 575.5 and 543 nm (Weber & Broadbent, 1975). Addition of azetidine-2-carboxylic acid at 7.3 μM , i.e. 3.2-fold above its K_a (see Methods), caused a transient increase in absorption at 557 nm compared with absorption at either 575.5 or 543 nm (Fig. 1). Proline itself (3.2 μM) had the same effect. Either proline or azetidine-2-carboxylic acid each at its K_m concentration caused smooth swimming for several seconds, after which normal swimming and tumbling resumed.

Fig. 1. Effect of attractant on absorption of cytochrome *b*. Attractant was added at the times noted by the arrows. Other discontinuities indicate earlier stirrings. (a) $A_{557} - A_{575.5}$, attractant was $7.3 \mu\text{M}$ -azetidine-2-carboxylic acid. (b) $A_{557} - A_{543}$, attractant was $2.3 \mu\text{M}$ -azetidine-2-carboxylic acid.

Fig. 2. Effect of lack of motility. Attractant ($7.3 \mu\text{M}$ -azetidine-2-carboxylic acid) was added at the times noted by the arrows. $A_{557} - A_{575.5}$. (a) 01401, a straight-flagella mutant. (b) Wild type, $0.2 \text{ mM-LaN}(\text{O}_3)_3$ added before tracing. (c) sc3, a straight-flagella mutant. (d) sc4, a straight-flagella mutant.

Fig. 3. Effect of deprivation of added cations. Buffer (see Methods) was used at 80 % strength but KSCN was omitted. Attractant ($10 \mu\text{M}$ -proline) was added at the times noted by the arrows. $A_{557} - A_{575.5}$. (a) Wild type. (b) 01401, a straight-flagella mutant.

Requirements for absorption change. Good motility was necessary for reduction of cytochrome *b* (measured as the difference between absorption at 557 nm and absorption at 575.5 nm). If a non-motile strain (straight, rotating flagella) was used, no reduction was observed (Fig. 2). If $0.2 \text{ mM-LaN}(\text{O}_3)_3$ was added to wild-type bacteria, making them non-motile, there was no reduction. If sufficient monovalent or divalent cation was omitted, there was no reduction (see below).

Requirement of cations. If the basic buffer, consisting of 0.8 mM-PIPES , 0.08 mM-lactate and 0.08 mM-EDTA , final pH 6.5, was not further supplemented, no absorption increase was observed. In fact, with wild-type bacteria, the act of stirring produced a decrease of absorption, i.e. cytochrome oxidation (Fig. 3). These bacteria were non-motile or only very sluggishly motile. Such a decrease was not observed for a straight-flagella mutant (Fig. 3). However, addition of divalent cations at low concentrations or monovalent cations at high concentrations afforded both good motility and cytochrome *b* reduction (Fig. 4; Table 1). Motility of bacteria soon after addition of attractant was examined

Fig. 4. Thresholds for added cations to restore attractant-induced cytochrome *b* reduction. Buffer (see Methods) was used at 80% strength (without KSCN) and salts were added at the concentrations indicated beside the traces. pH 6.5 to 6.6. Attractant (10 μM -proline) was added at the times noted by the arrows. $A_{557} - A_{575.5}$.

Table 1. *Threshold concentrations of cations for cytochrome b reduction*

Data taken from Fig. 4. The 'threshold concentration' is the minimum concentration of the salt that had to be added to the basic buffer of PIPES, lactate and EDTA to afford attractant-induced cytochrome *b* reduction. No reduction was observed when the salt concentration was decreased 1.8-fold.

Salt	Threshold concn
BaCl ₂	25 μ M
CaCl ₂	25 μ M
MgCl ₂	0.14 mM
CsCl	8.0 mM
KCl	8.0 mM
KSCN	14 mM
K ₂ SO ₄	14* mM
NaCl	25 mM

* In potassium equivalents: in true molarity, the threshold concentration is 7.0 mM.

Fig. 5. Cytochrome *b* reduction as a function of azetidine-2-carboxylic acid and leucine concentration. Attractant (at the concentrations indicated beside the traces) was added at the times noted by the arrows. $A_{557} - A_{575.5}$.

microscopically. At the threshold concentration, motility was satisfactory, but it was not excellent unless threefold higher concentrations were used. At sub-threshold concentrations it was significantly impaired.

The results summarized in Table 1 indicate (i) that the main effect on threshold is due to the cation, not the anion, (ii) that divalent cations are more potent than monovalent cations and (iii) that higher congeners in the periodic table are more effective than lower congeners.

Table 2. *Threshold concentrations of attractants for cytochrome b reduction*

Data taken from Fig. 5 and from similar experiments. The 'threshold concentration' is the minimum concentration of the amino acid required to bring about cytochrome *b* reduction. No reduction was observed when the concentration was decreased 3.2-fold.

Amino acid	K_d^* (μM)	Threshold concn (μM)
Proline	1.0	0.10 ($= K_d/10$)
Azetidine-2-carboxylic acid	2.3	0.23 ($= K_d/10$)
Leucine	32	3.2 ($= K_d/10$)
Isoleucine	100	10 ($= K_d/10$)

* Values of K_d are from Ordal *et al.* (1977), except for azetidine-2-carboxylic acid for which the K_d value is from Ordal *et al.* (1978).

Threshold concentrations for cytochrome b reduction. The concentrations of attractants were varied to determine the minima (thresholds) at which cytochrome *b* reductions occurred. Figure 5 illustrates the absorption changes for azetidine-2-carboxylic acid and leucine. Table 2 gives the threshold values for several amino acids. In these instances the threshold appeared to be about $K_d/10$ although other (unreported) experiments sometimes indicated thresholds of $K_d/3.2$.

Addition of repellent. *Bacillus subtilis* is repelled by the uncoupler of oxidative phosphorylation trifluoromethoxycarbonylcyanidephenylhydrazine (FCCP) (Ordal & Goldman, 1975, 1976). Addition of $0.15 \mu\text{M}$ -FCCP simultaneously with $7.3 \mu\text{M}$ -azetidine-2-carboxylic acid prevented cytochrome *b* reduction but prior addition of FCCP did not (data not shown). Similarly, transient swimming (without tumbling) did not occur when repellent and attractant were added simultaneously but did occur when repellent was added before the attractant.

Addition of valinomycin. Valinomycin tends to clamp the membrane potential by catalysing electrogenic transfer of K^+ (Harold, 1972; Manson *et al.*, 1977). If added to bacteria in high concentrations of K^+ , it tends to reduce the membrane potential but not if added to bacteria in low concentrations of K^+ . Since a decrease of the proton electrochemical gradient, one component of which is membrane potential, brings about tumbling (Ordal & Goldman, 1976), one might expect valinomycin to cause transient tumbling and suppress attractant-mediated reduction of cytochrome *b*. Indeed, only when added simultaneously with attractant in high K^+ (56.3 mM -KSCN) did valinomycin ($5.6 \mu\text{g ml}^{-1}$) prevent attractant-induced ($7.3 \mu\text{M}$ azetidine-2-carboxylic acid) cytochrome *b* reduction. Valinomycin added earlier had no effect, nor when added simultaneously in the presence of high concentrations of Na^+ (56.3 mM -NaSCN) (data not shown).

Effect of methionine. Methionine prevents proline taxis (Ordal *et al.*, 1977). The methionine chemoreceptor is different from the proline chemoreceptor, and methionine prevents proline taxis by binding at the methionine receptor (Ordal *et al.*, 1977). Accordingly, methionine only blocked proline-induced ($3.2 \mu\text{M}$) cytochrome *b* reduction at concentrations above the methionine K_d (data not shown).

Effect of tetraphenylarsonium. Tetraphenylarsonium makes the bacteria swim perpetually (Ordal, 1976a). When added, it caused a transient cytochrome *b* reduction, and it prevented azetidine-2-carboxylic acid-induced cytochrome *b* reduction beyond that caused by stirring (data not shown). It also, for unknown reasons (but see Discussion), made the bacteria sensitive to stirring such that cytochrome *b* reductions seemed to occur after vigorous stirring (data not shown). Genetically non-motile bacteria (straight, rotating flagella) and wild type in $\text{La}(\text{NO}_3)_3$ sometimes showed the same effect.

DISCUSSION

Addition of attractant to a suspension of bacteria caused an increase of absorption at 557 nm compared with absorption at 543 and 575.5 nm. It is likely that this increase was due to reduction of cytochrome *b* since the absorption spectrum of this cytochrome in *B. subtilis* has peak and troughs at these wavelengths, respectively (Weber & Broadbent, 1975). However, until a mutant lacking cytochromes is secured and the effect is shown to be absent in this strain, such a conclusion is tentative. The signals, although small, were reproducible. This reproducibility is indicated by the two stirring events preceding addition of reagents in many of the figures: normally, there was no effect on absorption before addition of reagent.

The apparent cytochrome reduction on addition of attractant is clearly related to chemotaxis. (i) Good motility was needed. (ii) Attractants added at concentrations 10-fold below their respective K_a values brought about the response. (iii) Simultaneous addition of repellent prevented the response (and prevented swimming) but prior addition of repellent allowed the response. (iv) The presence of the dissociation constant concentration of methionine, which prevents proline chemotaxis (by binding at the methionine, not the proline, chemoreceptor; Ordal *et al.*, 1977), prevented the response. However, cytochrome reduction also occurred when bacteria were made to swim in other ways, such as by addition of tetraphenylarsonium. The connection between cytochrome reduction and the transition to exclusively swimming is reinforced by the observation that attractant added to bacteria already swimming (presence of tetraphenylarsonium) did not cause cytochrome reduction.

What does this cytochrome reduction mean? It is a sign that the H^+ electrochemical gradient (sum of membrane potential, interior negative, and pH gradient, interior alkaline), which by the chemiosmotic hypothesis (Harold, 1972) represents a store of energy, is diminished. The decrease of this gradient lowers the cytochrome potentials towards that of the $NAD^+/NADH$ couple, i.e. reduces them somewhat (Wilson *et al.*, 1974). Since respiratory rate is proportional to the concentration of reduced cytochrome a_3 and O_2 (Wilson *et al.*, 1974), respiration, and hence electron transport, speeds up to increase the rate of proton extrusion and restore the proton gradient. Once the proton gradient is restored, the rate of electron transport drops to a steady state.

Why is this energy consumed? Probably, swimming requires more energy than does tumbling because the flagella may tangle in tumbling and thus jam (arrest rotation) (Berg & Tedesco, 1975; Macnab, 1977; Macnab & Ornston, 1977). Bacteria lacking rotating flagella do not consume energy for motility. Flagella in straight-flagella mutants rotate normally (Silverman & Simon, 1974) but do not form bundles and hence consume the same energy when rotating clockwise as counter-clockwise. Poorly motile bacteria are presumed to consume too little additional energy, when attractant is added, to affect the redox state of cytochrome *b*.

One feature shared by non-motile bacteria [straight-flagella mutants or wild type poisoned by $La(NO_3)_3$] and exclusively swimming bacteria (presence of tetraphenylarsonium) was sensitivity to stirring. Often, some cytochrome reduction was observed. Bacteria harvested at lower densities were more sensitive (data not shown). The normally motile bacteria seemed less sensitive. However, the cytochrome reduction following addition of attractant, when the bacteria were all swimming, might also be due to sensitivity to stirring. One possible explanation is that stress on the bacteria, due to shear from stirring, causes some leakage of ions across the membrane, reducing the membrane potential (one component of the proton gradient) so that electron transport has to speed up somewhat to restore the proton gradient. Since decrease of the proton gradient causes tumbling (Ordal & Goldman, 1976), the drain on the proton gradient for motility will diminish and thus, at least partly, compensate for the energy lost from the leakage. This supposed tumbling might be referred to as 'thigmotaxis', i.e. sensitivity to touch.

An opposite effect (cytochrome oxidation) was shown by wild-type bacteria in the absence of added cations. The straight-flagella mutants did not show this effect. Hence, it may somehow relate to the ability of wild-type flagella to interact (tangle; jam).

Another mystery is why motility should require cations. It is known that motility is energized by transfer of protons, not other cations, into the cell (Manson *et al.*, 1977; Matsuura *et al.*, 1977). It may be that the cations are needed to reduce the potential at the surface of the plasma membrane (McLaughlin, 1977), possibly to avoid too much local acidity (McLaughlin, 1977), so that the proteins involved in motility can exist in the correct conformations. In this connection, motility in *B. subtilis* is impaired below pH 6 (van der Drift & de Jong, 1974).

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