Arsenate Arrests Flagellar Rotation in Cytoplasm-Free Envelopes of Bacteria

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The effect of arsenate on flagellar rotation in cytoplasm-free flagellated envelopes of Escherichia coli and Salmonella typhimurium was investigated. Flagellar rotation ceased as soon as the envelopes were exposed to arsenate. Inclusion of phosphate intracellularly (but not extracellularly) prevented the inhibition by arsenate. In a parallel experiment, the rotation was not affected by inclusion of an ATP trap (hexokinase and glucose) within the envelopes. It is concluded that arsenate affects the motor in a way other than reversible deenergization. This may be an irreversible damage to the cell or direct inhibition of the motor by arsenate. The latter possibility suggests that a process of phosphorylation or phosphate binding is involved in the motor function.

An observation made a number of years ago was that cytoplasm-free envelopes of Escherichia coli or Salmonella typhimurium, tethered to glass by their flagella, can be made to rotate clockwise (CW) by inclusion of purified CheY in them (16). At that time this observation was intriguing, because CW rotation in intact bacteria was known to require intracellular ATP (1, 3, 10, 17, 20); however, these envelopes rotated CW in the apparent absence of ATP. (When envelopes are prepared, the internal content of the bacterial cell is diluted at the lysis step by more than 104-fold; therefore, their ATP levels are presumably negligible.) To verify that the CW-rotating envelopes indeed did not contain residual ATP, we treated them with arsenate, an effective ATP-depleting agent in intact bacteria (10, 17). Surprisingly, arsenate immediately stopped not only the CW-rotating envelopes but also the counter-CW (CCW)-rotating, CheY-free envelopes. The purpose of this study is to discriminate between the potential reasons for the inhibition of flagellar rotation by arsenate. (It should be noted that even though the ATP requirement was later found to be for CheY phosphorylation [8, 9, 23] and thereby for increasing the binding of CheY to the switch [22], the question of why arsenate stopped the rotation of CheY-free envelopes has remained open.)

Inhibition of flagellar rotation by arsenate. We examined the effect of arsenate in both CCW (CheY-free) and CW (CheY-containing) envelopes. Envelopes were isolated from S. typhimurium ST1 (2) and from E. coli RP437 (12) (both strains are wild type for chemotaxis) by penicillin treatment and subsequent osmotic lysis as previously described (14). CheY-containing envelopes were prepared by inclusion of 50 μM CheY (overproduced and purified essentially as described by Matsumura et al. [11] with the modifications described by Barak and Eisenbach [4]) in the lysis medium (16). Flagellar rotation was assayed at 25°C by the tethering technique (19) as previously described (13), using a flow chamber (6). (It should be noted that each envelope was separately tested for lack of cytoplasmic remnants as previously described [15]. Envelopes which were not verified to be cytoplasm and energy depleted were not included in the study.) Rotation was initiated by the addition of the respiratory substrate d,l-lactic acid (2 mM) to the flow medium. Exogenous addition of arsenate (on top of the lactic acid) stopped the rotation of both CCW (Fig. 1) and CW (not shown) envelopes within 2 min. Removal of arsenate from the flow medium caused resumption of rotation, provided that the incubation time with arsenate was relatively short: about 67% of the envelopes resumed their rotation when the incubation time was 5 min or less, but only about 17% did so when the incubation time was 8 min or longer. The inhibitory effect of arsenate was much reduced when phosphate was included within the envelopes (Fig. 1), indicating (i) that arsenate acts in the envelopes as a competitive inhibitor of phosphate and (ii) that its site of action is intracellular. It should be noted in this regard that E. coli has two primary P; transport systems: the pst system which is ATP dependent, having a higher specificity for phosphate than for arsenate, and the pit system, which is a proton motive force [PMF]-dependent system having similar affinities for phosphate and arsenate [18]. The pit system, which is a constitutive system, is probably functional in envelopes energized by lactate.

Potential causes of the inhibition. Since the rotation of the motor is driven by a PMF and the latter is generated by the respiratory electron transport, the arsenate-dependent inhibition of rotation could result from an inhibitory effect of arsenate on the electron transport, on the PMF, or on the motor itself.

Electron-transport system inhibition? To examine the first possibility, i.e., to determine whether arsenate affects the electron transport system directly (not via ATP synthesis), we measured oxygen uptake by envelopes in the presence and absence of arsenate. The measurement was carried out, essentially as described earlier (7), at 30°C with a Rank Brothers digital oxygen electrode system (model 10), using d,l-lactic acid as the energy source. The rate of the oxygen uptake was 38 ± 5 nmol of O2 per min in all cases, indicating that a direct effect of arsenate on the electron transport system does not occur.

Arsenate interferes with ATP synthesis by causing futile cycles of synthesis and spontaneous hydrolysis of ADP-arsenate (10). This is how it depletes the cells of ATP, but this is also how the futile cycles of ATP synthase can lead to PMF dissipation (10, 17). (Flagellar rotation in envelopes requires a
PMF of ≥29 mV [15].) We examined ATP depletion and PMF dissipation as potential causes of flagellar arrest.

**ATP depletion?** To examine the effect of ATP depletion, we prepared envelopes that contained (instead of arsenate) an ATP trap, consisting of hexokinase and glucose (24). In this case the lysis medium consisted of Tris-HCl (50 mM, pH 7.9), hexokinase (10 U/ml), glucose (25 mM), MgSO₄ (2 mM), and Tetren (tetraethylenepentamin 0.1 mM), both with or without ADP (5 mM) and bovine serum albumin (0.5 mg/ml). The flow medium for these envelopes consisted of Tris-HCl (50 mM, pH 7.9), D,L-lactic acid (2 mM), and Tetren (0.1 mM). The trap-containing envelopes rotated just as well as trap-free envelopes, indicating that ATP depletion per se does not arrest the rotation. (We verified the efficiency of hexokinase and glucose as an ATP trap under the same conditions by measuring ATP hydrolysis at increasing ATP concentrations.) This suggests that the reason for the cessation of rotation in the presence of arsenate was not ATP depletion. This conclusion is in line with the published observation that envelopes containing high levels of ADP (i.e., envelopes with a low phosphate potential) rotate as well as ADP-free envelopes (4).

**PMF dissipation?** We examined PMF dissipation as the cause of flagellar arrest by indirect means, because the PMF cannot be measured in envelopes due to their extreme fragility (7). Our criterion was the reversibility of the arsenate effect. We anticipated that if PMF dissipation was the cause, the inhibition of rotation by arsenate in envelopes would be fully reversible, i.e., flagellar rotation would resume upon arsenate removal (and thereby, PMF restoration by the respiratory system). This was not the case. Reversibility was observed only when the incubation time with arsenate was shorter than 5 min; no reversibility was observed after longer periods of incubation. Furthermore, this lack of reversibility was not the result of some cell damage due to energy depletion. This is because envelopes can remain in the complete absence of PMF for at least 5 h and be functional thereafter. Thus, freshly prepared envelopes are already depleted of energy, and their flagella do not rotate. They can undergo several cycles of energization (e.g., by addition of D,L-lactic acid) and deenergization (by substrate removal) with concomitant rotation or cessation of rotation, respectively (15).

**Conclusion.** The results of this study indicate that the cessation of rotation by arsenate is not the consequence of inhibition of the electron-transport system, ATP depletion, or reversible dissipation of PMF. Other potential causes are still open. These include irreversible dissipation of PMF, an irreversible damage to the cell, or direct motor inhibition by arsenate. If the latter possibility holds, it suggests that phosphate groups are involved in the motor function. These may be phosphate-binding sites or phosphorylation sites on the cytoplasmic side of the switch-motor complex or phosphorylation sites on a protein which binds to this complex. Accordingly, the rotation ceases when arsenate binds to these sites or prevents the phosphorylation. A similar phenomenon occurs also in intact cells, though with a 2-orders-of-magnitude-longer delayed response time: ≈2 h of incubation with arsenate is required for cessation of rotation (10, 21). This longer lag may be due to the presence of phosphate ions in intact cells.

It is of interest to note in this connection that the sweet-tasting protein thaumatin appears to be functionally similar to arsenate: it completely blocks the motility of E. coli, but the motility is restored by the addition of phosphate (5).

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**REFERENCES**


