$_{\Delta}\bar{\mu}H^{+}$ is required for flagellar growth in *Escherichia coli*

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Received 24 May 1982

Bacterial flagella *Protein transport* *Membrane potential*

1. INTRODUCTION

Growth of bacterial flagella has attracted considerable attention as a simple model system for studying biogenesis of a cell component (reviewed in [1,2]). Flagella are built of a simple monomeric protein, flagellin, and can be composed by self-assembly in vitro [1]. Flagella grow at their distal-end and new flagellin molecules must therefore pass from the cytoplasm to the pore in the filament and finally reach the tip of the organelle. This complex transport process must be energy-dependent [1]. Studying ubiquinone-deficient *Escherichia coli* mutants, flagellar growth was found suppressed and it was suggested that the dependence of flagellar formation on the protonmotive force could cause the defect [3]. Efficient flagellar growth proceeded in cells with either aerobic or anaerobic electron transport [3,4]. However, it was subsequently concluded that ubiquinone had a specific role in flagellar biogenesis, the process being independent of $_{\Delta}\bar{\mu}H^{+}$, and have attributed the lack of flagella in *ubi*-mutants to a decrease in the intracellular level of cyclic AMP [5,6]. Thus, the early observation [7] that flagellar growth in *S. typhimurium* was completely inhibited by dinitrophenol, but not by arsenate or azide, remains uninterpreted.

Here, we report that flagellar growth in *E. coli* is $_{\Delta}\bar{\mu}H^{+}$-dependent and apparently utilizes the energy of both the electrical and chemical components of $_{\Delta}\bar{\mu}H^{+}$.

Abbreviations: $_{\Delta}\bar{\mu}H^{+}$, transmembrane electrochemical proton potential difference; TPP^{+}, tetraphenylphosphonium cation; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole

2. MATERIALS AND METHODS

2.1. *Bacterial growth*

Escherichia coli AN 120 [8] was grown in minimal salt medium containing (per liter): KH_2PO_4, 13.6 g; $(NH_4)_2SO_4$, 2 g; KNO_3, 0.5 g; NaCl, 0.5 g; Na-citrate, 0.5 g; $MgSO_4 \cdot 7\ H_2O$, 0.25 g; $FeSO_4$, 0.5 mg; CaCl, 0.5 mg; and supplemented with: arginine, 20 mg/l; thiamine, 5 mg/l; streptomycin, 100 mg/l, and 54 mM glycerol; pH was adjusted to 7.0 with KOH. Cells were harvested in the late exponential phase of growth and resuspended in the basal salts medium containing additionally 100 μg chloramphenicol/ml. The suspension was passed through a microsyringe needle until all the cells lost motility as observed under phase contrast. Immotile cells were washed in chloramphenicol-containing medium and then incubated in the basal salts medium containing 22 mM glucose, 100 μg/ml chloramphenicol and appropriate effectors at 37°C for 3 h.

2.2. *Assays*

For determination of the number of flagella formed the cells were fixed with 2% formaldehyde, applied on Formvar-coated copper grids and round-shadowed with palladium, following [9]. The cells were observed under a Hitachi HU-11B electron microscope operating at 75 kV; 100 cells were counted for each experimental point.

The cellular ATP content was measured with a purified luciferin–luciferase preparation [10] by means of a 'Pico-ATP' luminometer (Jobin Ivon) after dimethylsulfoxide extraction [11] as described previously (I.I. Brown, M.Yu.G., A.N.G., V.P. Skulachev, submitted). Bacterial inner volume was

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

Table 1

Energy requirements of flagellar formation in *E. coli* AN120

Addition	No. flagella/ 100 cells	ATP (mM)	Membrane potential (mV)
None	95	2.8	130
2×10^{-5} M TTFB	8	2.8	< 30

Flagella from *E. coli* AN120 (8×10^8 cells/ml) were gently removed and were allowed to regenerate in a salt medium containing 22 mM glucose as energy source and 100 μg/ml chloramphenicol. After 3 h incubation flagella were counted under electron microscopy, ATP was determined with firefly luciferase, membrane potential was measured with a TPP^+-sensitive electrode. The results given are means of ≥ 3 determinations.

calculated from the *A*-values using the coefficients given in [12].

Membrane potential of EDTA-treated cells was measured by TPP^+ accumulation with a TPP^+-sensitive membrane electrode [13], kindly provided by Dr L. Grinius. The incubated cells were collected by centrifugation and treated with 5 mM EDTA [12]. A portion of the suspension obtained was injected into the chamber containing the same medium and 3 μM TPP^+. Boiled cells were used as a control of the amount of TPP^+ bound in an energy-independent manner. For further details see [13].

2.3. *Reagents*

The luciferin–luciferase preparation used was the 'ATP monitoring reagent' from LKB-Wallac, dimethylsulfoxide was from Koch-Light, chloramphenicol from Calbiochem, EDTA, *p*-fluorophenylanaline and puromycin from Serva, cyclic AMP, ATP and valinomycine from Sigma. Nigericin was a kind gift from Dr M. Baltscheffsky. All other chemicals were of reagent grade.

3. RESULTS AND DISCUSSION

The energy source for flagellar growth in *E. coli* was studied using an AN120 strain that lacks a functional H^+-ATPase [8] and is thus unable to interconvert the energy of $\Delta\bar{\mu}H^+$ and ATP. The flagella were gently removed by passing the cell suspension through a microsyringe needle and then allowed to grow again.

Cells incubated without any uncoupler were found to regain motility even in the presence of chloramphenicol (table 1). Sheared cells incubated with an uncoupler lacked flagella. The lack of flagellar growth could not be attributed to a decreased ATP level, since ATP concentration appeared to be the same in the presence and in the absence of the uncoupler (table 1).

The observed formation of flagella in the presence of chloramphenicol pointed to the existence of a preformed pool of flagellin, described for *E. coli* [14] and established in several other species (see [15–17], discussed in [2]). Several lines of evidence were obtained substantiating the finding of flagellar formation from presynthesized flagellin molecules. The growth of flagella was not inhibited by chloramphenicol or puromycin and occurred in the absence of exogenous amino acids. Though flagellin containing *p*-fluorophenylalanine is known to produce flagella with curly waveform [1], no curly flagella were observed when flagella regenerated in the presence of 1 mM *p*-fluorophenylalanine. The additon of an uncoupler to the growth medium resulted in impaired formation of flagella thus permitting an accumulation of a greater pool (not shown). Taken together these facts strongly suggest that newly formed flagellin molecules indeed enter a functional pool and are then transferred to the point of insertion into a growing flagellum.

The analysis of the relative contributions of electrical and chemical components of $\Delta\bar{\mu}H^+$ revealed that both components seem to be capable of energizing flagellar formation (table 2). TPP^+ which

Table 2

Membrane potential and the pH difference in supporting flagellar formation

Addition	No. flagella/ 100 cells
None	101
TPP^+	68
Acetate	57
TPP^+ + acetate	10
TPP^+ + acetate + cAMP	12

Flagella were sheared and the cells were incubated for 3 h in the presence of: TPP^+ chloride, 250 μM; sodium acetate, 50 mM; cyclic AMP, 2 mM.

lowers the membrane potential or acetate which diminishes the transmembrane pH difference were found to partially suppress the growth of flagella when added separately. A combination of TPP^+ and acetate completely inhibited flagellar formation. Cells did not escape the uncoupler action even in the presence of cyclic AMP (table 2) shown to activate flagellar formation when added to a respiration-deficient mutant of *E. coli* [6]. The flagellar formation in EDTA-treated cells, hardly noticeable as it is, was completely inhibited when valinomycin or nigericin were added for selective lowering of either the membrane potential or the pH difference. This can be attributed to the lowered $\Delta\bar{\mu}H^+$-values in the presence of these antibiotics. Indeed, a rather high threshold in the $\Delta\bar{\mu}H^+$-values needed to support flagellar formation is suggested by the fact that $\Delta\bar{\mu}H^+$ generated by the H^+-ATPase under strictly anaerobic conditions was found insufficient for supporting the process [3,4]. An insufficient $\Delta\bar{\mu}H^+$ level could also be the reason for an impaired flagellar growth at high temperatures, as observed in [16].

The dependence of protein (flagellin) translocation on the pH difference found here is a strong indication of the existence of a specific flagellin translocase. To couple the membrane potential to flagellar assembly, it would be sufficient to allow the negatively charged flagellin molecules to move across the membrane by self-electrophoresis. However, to use the energy of pH difference, there must be an inward flow of protons, accompanying flagellin transport. It seems that the coupling of opposed fluxes of flagellin and H^+ would inevitably require a specific translocase.

Uncouplers were demonstrated to inhibit maturation of several proteins and their insertion into the membrane or into the periplasmic space [18–21]. Though the direct effects of uncouplers on the bacterial ATP content and, consequently, on the ATP-stimulated protease activity [22] were excluded only in the case of phage M13 coat protein [18], it seems likely that membrane potential was indeed required for the effective protein translocation. The role of $\Delta\bar{\mu}H^+$ is, however, generally attributed to its effect on the formation of some unique protein conformation that allows effective cleavage of a signal segment (see [23,24] for discussions). The results presented here stimulate analysis of the possibility of an additional role of $\Delta\bar{\mu}H^+$ as the energy source for protein translocation, a 'protein driving force'. This idea is further substantiated by the similar results obtained for the energization of the incorporation of cytoplasmically synthesized aspartate aminotransferase into mitochondria [25].

ACKNOWLEDGEMENTS

The authors are grateful to Dr L. Grinius for a kind gift of the TPP^+-electrode, to Dr G. Gudima for the help in electron microscopy and to Professor V.P. Skulachev for helpful discussions.

REFERENCES

[1] Iino, T. (1969) Bacteriol. Rev. 33, 454–475.
[2] Kerridge, D. (1973) in: The Generation of Subcellular Structures (Markham, R. et al. eds) pp. 135–165, Elsevier Biomedical, Amsterdam, New York.
[3] Bar-Tana, J., Howlett, B.J. and Koshland, D.E. jr (1977) J. Bacteriol. 130, 787–792.
[4] Hertz, R. and Bar-Tana, J. (1977) J. Bacteriol. 132, 1034–1035.
[5] Bar-Tana, J., Howlett, B.J. and Hertz, R. (1980) J. Bacteriol. 143, 637–643.
[6] Hertz, R. and Bar-Tana, J. (1982) Arch. Biochem. Biophys. 213, 193–199.
[7] Kerridge, D. (1960) J. Gen. Microbiol. 23, 519–538.
[8] Butlin, J.D., Cox, G.B. and Gibson,F. (1971) Biochem. J. 214, 75–81.
[9] Jacobson, A. (1972) J. Virol. 10, 835–843.

[10] Lundin, A., Rickardson, A. and Thore, A. (1976) Anal. Biochem. 75, 611–620.
[11] Jakubozac, E. and Leclerc, H. (1980) Ann. Biol. Clin. 38, 297–304.
[12] Kashket, E.R. (1981) J. Bacteriol. 146, 377–384.
[13] Grinius, L.L., Dangelavicius, G.A. and Alkimavicius, G.A. (1980) Biokhimiya 45, 1609–1618.
[14] Vaituzis, Z. and Doetch, R.N. (1966) J. Bacteriol. 91, 2103–2104.
[15] Kerridge, D. (1963) J. Gen. Microbiol. 33, 63–76.
[16] Martinez, R.J. and Gordee, E.Z. (1966) J. Bacteriol. 91, 870–875.
[17] Smith, R.W. and Koffler, H. (1971) Adv. Microbiol. Physiol. 6, 219–339.
[18] Date, T., Zwizinsky, C., Ludmerer, S. and Wickner, W. (1980) Proc. Natl. Acad. Sci. USA 77, 827–831.
[19] Date, T., Goodman, J.M. and Wickner, W. (1980) Proc. Natl. Acad. Sci. USA 77, 4669–4673.
[20] Enequist, H.C., Hirst, T.R., Hardy, S.J.S., Harayama, S. and Randall, L.L. (1981) Eur. J. Biochem. 116, 227–233.
[21] Daniels, C.J., Bole, D.G., Quay, S.C. and Oxender, D.L. (1981) Proc. Natl. Acad. Sci. USA 78, 5396–5400.
[22] Voellmy, R.W. and Goldberg, A.L. (1981) Nature 290, 419–421.
[23] Waksman, A., Hubert, P., Cremel, G., Rendon, A. and Burgun, C. (1980) Biochim. Biophys. Acta 604, 249–296.
[24] Inouye, M. and Halegoua, S. (1979) Crit. Rev. Biochem. 7, 339–371.
[25] Passarela, S., Marra, E., Doonan, S., Languino, L.R., Saccone, C. and Quagliariello, E. (1982) Biochem. J. 202, 353–362.