Biochimica et Biophysica Acta, 686 (1982) 19–26
Elsevier Biomedical Press

BBA 71100

METHYL-ACCEPTING CHEMOTAXIS PROTEINS ARE DISTRIBUTED IN THE MEMBRANE INDEPENDENTLY FROM BASAL ENDS OF BACTERIAL FLAGELLA

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(Received September 15th, 1981)

Key words: Chemotaxis; Membrane protein; Methyl-accepting chemotaxis protein; Bacterial flagella; (Random distribution, E. coli)

Chemotactic behavior of *Escherichia coli* involves communication between methyl-accepting chemotaxis proteins and basal ends, the rotary motors of bacterial flagella. Both the proteins and the basal ends are embedded in the cytoplasmic membrane, but the spatial relationship between the two has not been determined. This communication describes a procedure for obtaining a preparation of membrane vesicles enriched in basal ends and thus in the regions of membrane immediately surrounding them. Methyl-accepting chemotaxis proteins were neither enriched nor depleted in this membrane fraction but instead were distributed throughout the membrane. Thus functional linkages between these proteins and flagellar motors must be mediated by processes other than direct physical interaction.

Introduction

Motile strains of *Escherichia coli* contain a sensory system which allows cells to migrate to favorable chemical environments; that is, to perform chemotaxis [1,2]. Specific receptors on the cell surface recognize chemotacticly active compounds. Changes in the proportion of receptors which are ligand-ocupied result in alterations in the functioning of the flagellar motor, producing net migration of a cell in a chemical gradient. Chemotactic behavior can be divided into two processes, excitation and adaptation. The features of the two are well illustrated by considering the sequence of events which occur when cells are subjected to a temporal gradient; for example, addition of an attractant compound to a cell suspension. In unstimulated cells, flagellar motors alternate between counterclockwise and clockwise rotation. Immediately (< 1 s) after attractant is added, all motors respond by rotating exclusively in a counterclockwise direction. This is excitation. It requires a specific receptor site on the cell surface [3], communication of site occupancy through an integral membrane protein transducer [2,4] and transfer of a 'chemotactic signal' from transducer to the flagellar motor [2,4] which is also embedded in the cytoplasmic membrane. The nature of the link between transducer and motor is not yet understood.

Several seconds to several minutes after attractant addition, depending on the nature and the magnitude of the stimulation, the initial pattern of counterclockwise and clockwise flagellar rotation is reestablished. This is adaptation [1]. It is directly correlated with changes in the level of carboxyl methylation of specific glutamic acid residues in the transducer protein through which excitation occurred [4]. This change is an increase in the level of methylation for adaptation to favorable stimuli such as attractant addition. Because transducers are substrates for the methylation reactions, they are called methyl-accepting chemotaxis proteins

Abbreviation: MCP or acceptor protein, methyl-accepting chemotaxis protein.

(MCP or acceptor protein). Each transducer/acceptor protein is related to a group of receptors. There are approximately twenty different types of receptors [3], but many fewer transducers, probably four or five [4,5]. The genes coding for MCP I, II and III are *tsr*, *tar* and *trg*, respectively [6–9]. MCP I and II account for about 90% of the methyl -accepting proteins found in the membrane of wild-type cells [10].

The flagellar motor is a complex of disks and rods [11] constructed from a total of at least nine different proteins [12,13]. The structure, called a basal end, rotates a rod which is connected to a hook which serves as a universal joint, and is in turn connected to the flagellar filament, a long, left-handed helical polymer of the protein flagellin. In *E. coli*, about six basal ends are randomly distributed over the cell surface but the filaments can rotate as a single bundle as a result of appropriate bends in the respective hooks [14]. If acceptor proteins were intimately associated with basal ends, then it would be possible that functional linkages between transducer and motor would be accomplished by direct physical interactions. If acceptor proteins were not localized in the regions of membrane surrounding basal ends, then transfer of the excitatory signal by direct contact would be unlikely. Acceptor proteins are not among the proteins found in isolated basal ends [12,13]. However, isolation of basal ends from cell envelope involves dissolution of cellular membranes with detergent and thus it is quite possible that cytoplasmic membrane proteins associated with basal structures are released by the isolation procedure. Such proteins might include the *mot* gene products, necessary for flagellar rotation [12,13], as well as transducer/acceptor proteins.

In this communication we describe a procedure for obtaining a preparation of membrane vesicles from *E. coli* enriched in basal ends and thus in the regions of membrane immediately surrounding flagellar motors. This preparation allowed us to determine the localization in the membrane of methyl-accepting chemotaxis proteins relative to basal ends. Acceptor proteins were neither enriched nor depleted in the membrane fraction enriched in basal ends but instead were distributed throughout the membrane.

Methods

Strains. Escherichia coli K12 strains HB1 and HB2 are this laboratory's copies of HfrG6 and pop1048, obtained originally from M. Schwartz, Institut Pasteur, Paris. Both were used routinely for preparation of flagella vesicles. Preparations have also been done using SH7 [15]. We expect that flagellar vesicles could be prepared from any flagellated strain.

Media. The growth media, containing tryptone or minimal salts (H1), required amino acids and a carbohydrate source, have been described previously [10].

Radioisotopes. L-[*Me*-^{3}H]Methionine (70–85 Ci/mmol) and L-[^{35}S]methionine (> 1000 Ci/mmol) were purchased from New England Nuclear Corp.

Preparation of flagellar vesicles. This is a modification of the procedure developed by Randall and Hardy [15] for the preparation of membrane-bound polysomes. Routinely, 1 liter of complex or minimal medium was inoculated with highly motile cells to a density of about $2.5 \cdot 10^7$ cells/ml and grown at 35°C with vigorous aeration on a rotary shaker to approx. $5 \cdot 10^8$ cells/ml. The cells were harvested by centrifugation for 1.5 min at 7500 rev./min in a GS-3 rotor (Sorvall), resuspended to a final volume of 16 ml in ice-cold chemotaxis buffer (10 mM potassium phosphate (pH 7), 1 mM EDTA) and additional EDTA added to give a final concentration of 6 mM. This suspension was divided into two portions, each placed in a 15 ml scintillation vial and then disrupted while cooled in an ice-salt bath by two 6-s periods of ultrasonic vibration using a standard 8 mm diameter probe. This treatment breaks over 90% of the cells. DNAase was added to a final concentration of 10 μg/ml and the suspension layered on top of six 5–28% (w/w) sucrose gradients (27.5 ml) in chemotaxis buffer over an 8 ml 56% sucrose cushion in the same buffer in 38.5 ml capacity cellulose nitrate tubes. The gradients were centrifuged 90 min at 27000 rev./min using an SW27 rotor and collected in approximately 20 fractions using an ISCO gradient fractionator. The flagellin-rich zone was located by SDS-polyacrylamide gel electrophoresis as described in Fig. 1. Appropriate fractions were then pooled, diluted to 5% sucrose and centrifuged

for at least 6 h at 100000 × *g*. Pelleted material contained flagellar fragments, flagellar vesicles and other membrane vesicles as discussed in Results.

SDS polyacrylamide gel electrophoresis. The method was that of Laemmli [16] with the modifications described by Randall and Hardy [15]. Gels prepared for fluorography were treated with En^{3}Hance (New England Nuclear Corp., Boston, MA), dried and exposed to X-ray film at −70°C. Otherwise, gels were dried and analyzed by autoradiography directly.

Results

Flagellar vesicles

Brief sonication of moderately dense suspensions of cells of *Escherichia coli* results in breakage of approx. 90% of the cells into large pieces of envelope and small membrane vesicles. When this mixture is placed on top of a 5 to 28% (w/w) gradient of sucrose over a layer of 56% sucrose and centrifuged under appropriate conditions, the large envelope fragments sediment to the boundary between the cushion of 56% sucrose and the gradient, small membranes vesicles and ribosomes are distributed within the gradient and soluble protein remains at the top [15]. We found that a slightly modified form of this procedure, performed on motile cells, allowed separation of flagellar fragments from the bulk of other cellular material. This fraction included stubs of flagella attached to small membrane vesicles, which we thus call flagellar vesicles.

Fig. 1 shows the distribution of proteins from motile cells in the preparation gradient. The presence of 6 mM EDTA in the suspension of broken cells dissociated ribosomes to the extent that those structures sedimented only slightly into the sucrose gradient and thus little protein was located in the middle of the gradient. A striking exception was the predominant, broad band at 55000 apparent molecular weight characteristic of flagellin [17,18]. The band was completely absent when non-motile, non-flagellated cells were subjected to the same procedure. The position of the flagellin zone was a function of the time of centrifugation. Electron

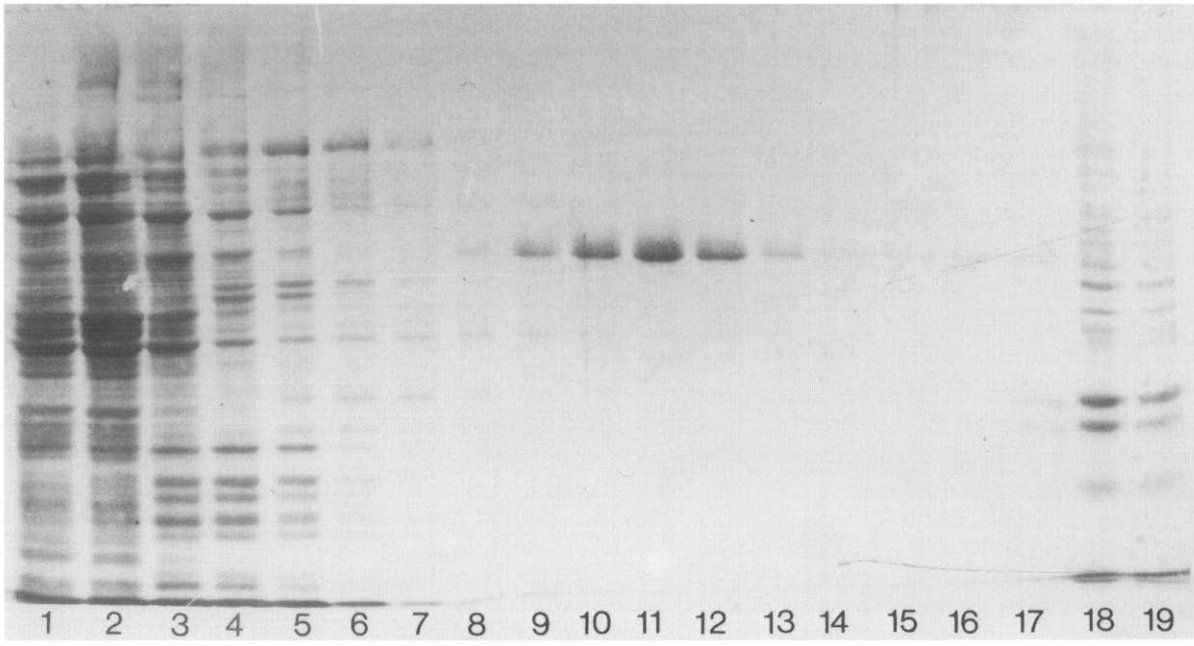

Fig. 1. Distribution of protein in gradient used for preparation of flagellar vesicles. 40 μl of the 1.8 ml fractions of a flagellar vesicle gradient containing materials from 10^{11} cells of HB1 were mixed with electrophoresis sample buffer, boiled for 3 min and applied to an SDS-polyacrylamide (10%) gel. After electrophoresis, the gel was stained with Coomassie brilliant blue. Flagellin is visible in fractions 9–13. Bulk membrane is in fractions 17–19.

micrographs of fractions rich in flagellin showed fragments of flagellar filaments of a relatively homogeneous size. We interpret these observations to mean that sonication breaks flagella into pieces which sediment through the sucrose gradient in a characteristic manner, resulting after any given time of centrifugation in a zone rich in flagellin.

Almost all [^{35}S]methionine-labeled protein (> 90%) in the zone containing flagellin was pelleted by subjecting gradient fractions diluted to 5% sucrose to centrifugation at 100 000 × g for more than 6 h, thus demonstrating that the protein was in a particulate form. Electron micrographs of negatively stained preparations of that material revealed a large number of flagellar fragments and a few membrane vesicles (Fig. 2). Most flagellar fragments were simply segments of filament, others included hook and filament and some hook-filament fragments were attached to small vesicles, the flagellar vesicles. In some cases, the outlines of a basal end could be seen at the junction of hook and membrane (Fig. 3) but the rings of the basal end were not always resolved in our negatively stained preparations. We distinguished flagellar vesicles from other vesicles that might have been positioned by chance near or in contact with a flagellar filament by the minimal criterion of an identifiable hook, which is slightly thicker and can be bent at much sharper angles than filament [19,20], protruding approximately normal to the

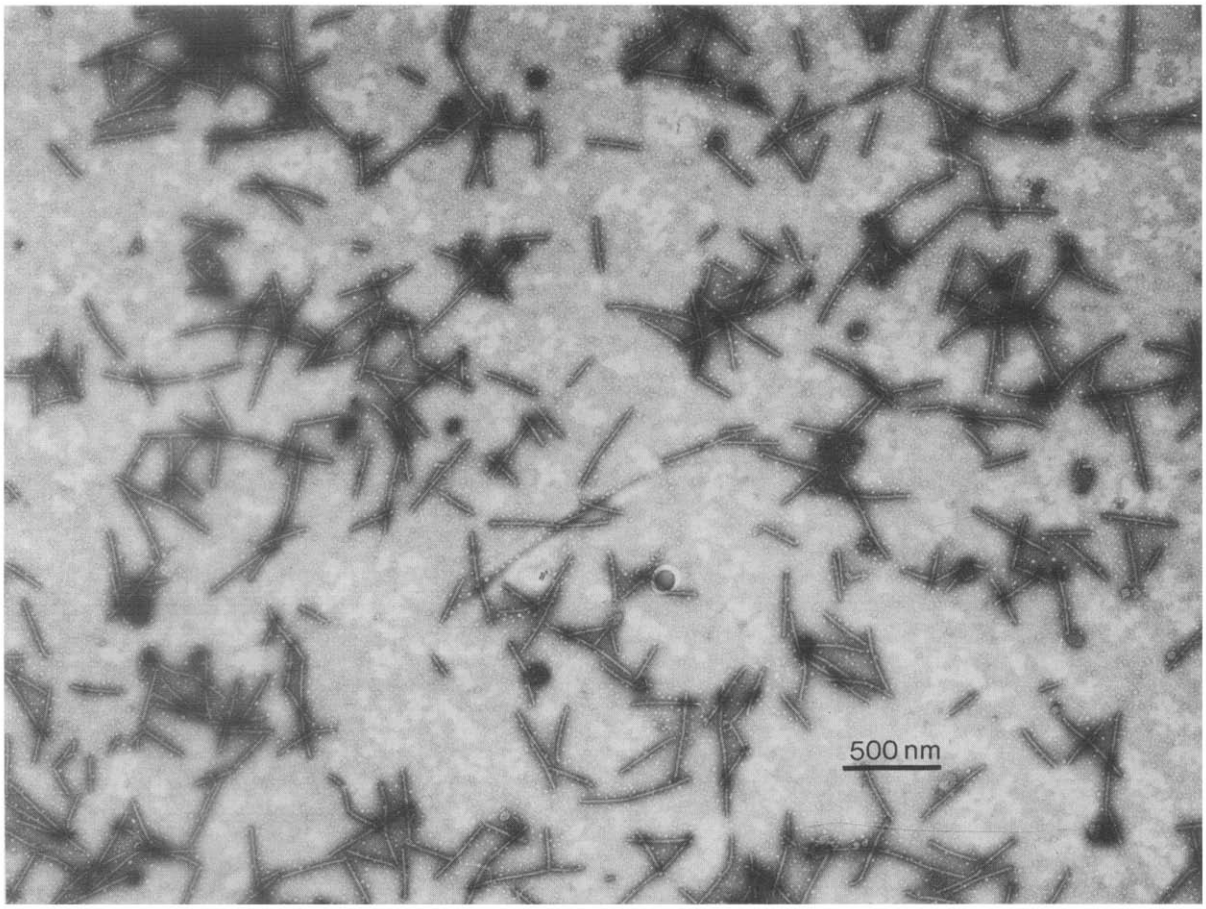

Fig. 2. Electron micrograph of particulate material contained in flagellar vesicle fractions. Fractions of a preparative gradient which contained flagellin (equivalent to fractions 9–13 in the gradient of Fig. 1) were pooled, diluted to 5% sucrose and centrifuged at 100 000 × g for 8 h. Pelleted material was resuspended in 100–200 μl of chemotaxis buffer and dialyzed against the same buffer. Samples were stained with 1% uranyl acetate, or 1% phosphotungstic acid.

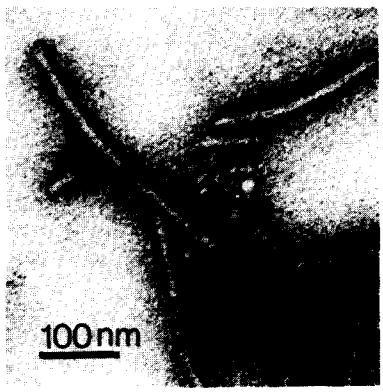

Fig. 3. A close-up view of a flagellar vesicle. Preparation was the same as for Fig. 2.

vesicle surface. Using this criterion we classified all the vesicles in many fields of view (>3000 vesicles total) and found that 18% were flagellar vesicles.

An important factor in the interpretation of studies of flagellar vesicles is the identity of the type of membrane which made up the vesicle. In whole cells, basal end rings are embedded in both the outer and the cytoplasmic membrane [20]. Either membrane can form a small vesicle enclosing the basal end [20,21] and thus the population of flagellar vesicles would probably include both cytoplasmic and outer membrane vesicles. That expectation was supported by the presence of the same mixture of cytoplasmic and outer membrane proteins in the flagellar vesicle fraction as in the bulk membrane (Fig. 4A). As another criterion, we utilized the differential solubilities of cytoplasmic and outer membranes to assay the population of membranes in the flagellar vesicle fraction. About 55% of [^{35}S]methionine-labeled membrane protein in the fraction was solubilized by Triton X-100 in the presence of 10 mM $MgCl_2$, a treatment that dissolves cytoplasmic membrane but not outer membrane [21,22]. Thus it appears that the ratio of cytoplasmic membrane to outer membrane is about the same in the flagellar vesicle fraction as it is for total membrane.

The flagellar vesicles were between 50 and 100 nm in diameter. For cells 2 μm by 1 μm with an average of six flagella per cell, the membrane required to form a vesicle of 75 nm diameter attached to each basal end would be 1.4% of the total cell surface area. Since vesicles are formed from both cytoplasmic and outer membranes, both of which encompass the total cell surface, 0.7% of total membrane would be required. The flagellar vesicle fraction contained about 3% of total membrane (see below and Table I) and 18% of the vesicles in that fraction were flagellar vesicles. Thus approx. 0.5% of total membrane was found in our preparations as flagellar vesicles. These figures indicate that the yield of basal ends was greater than 70% and that areas of cytoplasmic and outer membrane surrounding basal ends were each enriched at least 10-fold in the flagellar vesicle fraction. We used antiflagellin and antihook sera to attempt further enrichment by immune precipitation from the flagellar vesicle fraction.

TABLE I

DISTRIBUTION OF (*Me*-^{3}H)-LABELED PARTICULATE MATERIAL AMONG THE REGIONS OF A FLAGELLAR VESICLE GRADIENT

MCPs, methyl-accepting chemotaxis proteins.

Fraction	% of radioactivity	
	Total [d]	in MCPs [e]
Soluble proteins and ribosomes [a]	2	0.4
Flagellar vesicles [b]	3	2.6
Membrane [c]	95	97

[a] Corresponds to fractions 1–8, Fig. 1.

[b] Corresponds to fractions 9–13, Fig. 1.

[c] Corresponds to fractions 14–19, Fig. 1.

[d] Fractions of a gradient like that considered in Fig. 1 were pooled as indicated and centrifuged as described in the legend for Fig. 2. Samples of the material pelleted were taken and the amount of radioactivity determined using a scintillation counter with an efficiency of 85% for ^{3}H. As discussed in the text, this radioactivity is predominantly in cyclopropane fatty acids and thus is a good indicator of the relative amounts of membrane present. The total radioactivity in the gradient was about $3 \cdot 10^6$ cpm.

[e] Samples of the pelleted material described in (d) were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography as described in the legend to Fig. 4. Gel patterns like those shown in fig. 4B were traced using a Joyce-Loebl microdensitometer and the relative amounts of methyl-labeled acceptor protein in the different fractions were determined by cutting out and weighing peaks of densitometer tracings of appropriately exposed films, as described in Ref. 32.

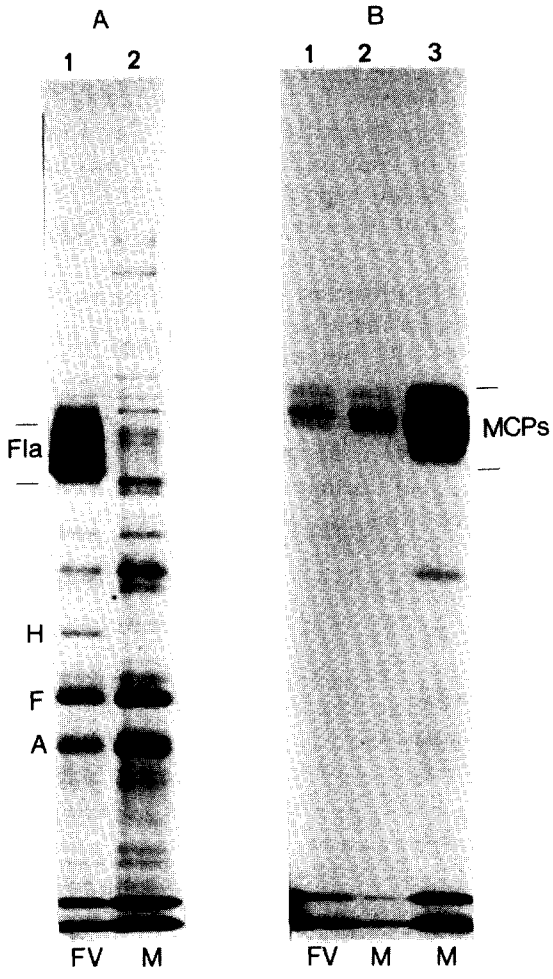

Fig. 4. Comparison of radioactively labeled protein found in the flagellar vesicle and bulk membrane fractions. Flagellar vesicles were prepared from parallel cultures of HB1 in minimal salts, ribose medium labeled with [^{35}S]methionine during growth or with [*Me*-^{3}H]methionine in conditions prohibiting protein synthesis but allowing protein and fatty acid methylation as described in Ref. 10. Fractions containing flagellin (equivalent to fractions 9–13 in Fig. 1) and bulk membrane (equivalent to fractions 17–19 in Fig. 1) were separately pooled and centrifuged as described in the legend to Fig. 2. Portions of the pelleted material were applied to an SDS-polyacrylamide (9%) gel. (A) Gel patterns of [^{35}S]methionine-labeled protein. To facilitate comparison of the proteins in the flagellar vesicle (F.V.) and bulk membrane (M) fractions, equal amounts of radioactive protein were applied to each lane. The M sample is only 1/55 of the proportion of the total M fraction that the F.V. sample is of the total F.V. fraction. The broad triplet bands of flagellin are indicated by 'Fla', the hook by an 'H' and the OmpA and OmpF proteins by an 'A' and 'F', respectively. The apparent molecular weights of those bands are Fla, 52000 –56000; H, 40000; A, 33000; F, 36000. The two dark bands at the bottom of the gel are artifacts of many low molecular weight proteins migrating in the ion fronts. (B) Gel patterns of [*Me*-^{3}H]methionine-labeled material. The figure is a fluorogram of material from flagella vesicle (F.V.) and membrane (M) fractions. See the text for explanation of the relative amount of sample in each lane. Methyl-accepting chemotaxis proteins are indicated by 'MCPs'. The two dark bands at the bottom of all three lanes are artifacts as described in (A). The fastest migrating band in lane 3 is phospholipid, (*Me*-^{3}H)-labeled as cyclopropane fatty acids [22,23]. That material is not quantitatively retained as the gels are processed.

The immune precipitates were not further enriched in flagellar vesicles, probably because the extensive precipitates trapped unrelated membrane vesicles.

A comparison of proteins found in the flagellar vesicle and bulk membrane fractions showed that flagellin and hook protein were found almost exclusively in the forner fraction (Fig. 4A). Hook protein was identified by immune precipitation by antihook serum [23]. Varying the length of autoradiogram exposure to gels like the one of Fig. 4A revealed that essentially all other bands visible in either lane were common to the two fractions. We would expect flagellar vesicles to be enriched for proteins of the basal end but since those proteins are present at very low copy number in whole cells [12,13], a 10-fold enrichment would not be sufficient to allow visualization of basal end protein bands among the many membrane proteins in the same fraction.

Distribution of methyl-accepting chemotaxis protein between membrane fractions

We used flagellar vesicle preparations to determine the localization of methyl-accepting chemotaxis proteins (acceptor proteins) relative to basal ends. If acceptor proteins were clustered near basal ends then the flagellar vesicle fraction, 10-fold enriched over bulk membrane in areas surrounding basal ends, would be similarly enriched for acceptor proteins. If acceptor proteins were not clustered then the concentration in a flagellar vesicle preparation would be the same as in bulk membrane.

Motile cells were incubated with [*Me*-^{3}H]methionine in conditions prohibiting protein synthesis but allowing methylation until incorporation of radioactive methyl groups by acceptor proteins reached a plateau. Under these condi-

tions, [^{3}H]methyl groups are transferred to a small number of proteins and to fatty acids (see below). Fig. 4B shows the pattern of pelleted, methyl-labeled protein in the flagellar vesicle and membrane fractions. The same quantity of radioactivity was applied to lane 1 (flagellar vesicles) and lane 2 (bulk membrane) in order to illustrate the similar patterns of methyl-labeled acceptor proteins in the two fractions. However, lane 1 contained 74% of the total flagellar vesicle material while lane 2 contained 1.5% of the bulk membrane. Increasing the amount of bulk membrane to 15% in lane 3 (one-fifth of the comparable proportion of total material in lane 1) causes overexposure of the fluorogram but illustrates that most of the acceptor proteins is not in the flagellar vesicle fraction, but rather in the same fraction as most of the membrane.

When cells are labeled with [*Me*-^{3}H]methionine in conditions prohibiting protein synthesis, over 90% of the radioactive methyl groups are incorporated not into proteins but rather as cyclopropane derivatives of unsaturated fatty acids contained in membrane lipids [24]. Essentially all lipids are susceptible to this derivatization and thus the methylation is a good general label for membrane. This means we could obtain reasonable estimates of the relative amounts of membrane in different fractions by determining the amount of total radioactive methyl groups present (Table I). The validity of this approach is supported by estimates of the relative amount of material in the flagellar vesicle fraction based on the distribution of other radioactive labels. For cells labeled with [2-^{14}C]-glycerol, which should be incorporated strongly into lipid and to some degree into protein, 3–5% of total pelleted radioactivity was in the flagellar vesicle fraction, values consistent with the one listed in Table I.

The relative amounts of methyl-labeled acceptor proteins were determined by densitometric tracings of appropriate fluorograms like the one shown in Fig. 4B, in which the density of the acceptor protein bands was within the dynamic range of the film. Table I illustrates that methyl-labeled acceptor proteins were found in the flagellar vesicle and bulk membrane fractions at levels proportional to the amount of membrane in those fractions. There was no indication that acceptor proteins were enriched in the membranes of flagellar vesicles, indicating that those proteins are not localized in membrane surrounding basal ends.

Discussion

The combination of brief sonication of motile cells and sedimentation of the resulting envelope fragments through a sucrose gradient allowed separation of almost all of the flagellin and hook from other cellular constituents. Such a separation can also be accomplished by shearing flagellar filaments from intact cells, removing the cells by low force centrifugation and subsequent banding of filaments in $CsCl_2$ [18,25]. The value of the procedure described here is that a significant number of hook-filament fragments were still attached to a basal end embedded in a small area of membrane which formed a vesicle upon being ripped from the cell envelope by sonication. Similar flagellar vesicles have often been observed in electron micrographs of broken cells and historically were the origin of some confusion about the basal structure of bacterial flagella [20]. However, there has not been a method available which allowed their isolation. The procedure described here produces a membrane fraction 10-fold enriched in these vesicle-flagellum structures.

The characterization of the flagellar vesicle fraction allowed us to investigate the distribution of acceptor proteins between membrane areas near basal ends and bulk membrane. Localization of all acceptor proteins, which are cytoplasmic membrane protein, near basal ends would have resulted in over 35% of those molecules found in the flagellar vesicle fraction, as calculated from the values of a 70% yield of basal ends and 50% of those basal ends attached to cytoplasmic membrane. The data in Fig. 4B and Table I show that only 3% of the acceptor proteins are in the flagellar vesicle fraction, indicating that acceptor proteins are not located exclusively in basal end regions. Quantitation of the amount of acceptor proteins relative to membrane lipid (Table I) indicates that acceptor proteins are distributed throughout the membrane, neither enriched not depleted in areas near basal ends. The validity of this conclusion rests on the assumption that flagellar vesicles contain the membrane components which surround basal ends in unbroken cells. Unfortunately, it is not possible

to test whether specific proteins are lost from flagellar vesicles during the preparation since there are no membrane proteins identified as associated with basal ends. However, it seems to us that the assumption is reasonable, since it is unlikely that a procedure which leaves most of the cellular membranes in very large fragments and functional membrane-bound polysomes attached to those fragments [15] would also result in complete reassortment between the proteins in flagellar vesicles and bulk membrane.

It has been argued that the most efficient way to distribute a small number of receptors over the surface of a cell is in a random distribution [26]. Acceptor proteins are both primary receptors for ligands [27,28] and secondary receptors for ligand-receptor complex [6–9], so it is reasonable that the distribution of acceptor proteins would be random, not localized in specific areas. A general distribution of acceptor proteins throughout the cytoplasmic membrane makes it unlikely that either excitation or adaption involves direct physical interaction between those molecules and basal ends, and instead implies that additional components or processes must link transducer/acceptor proteins and basal ends. There are indications in the literature that ion fluxes [29], calcium ions [30] and cyclic GMP [31] may participate in those linkages.

Acknowledgements

We thank Dr. L.L. Randall for bringing our attention to a predominant protein, which was flagellin, in the middle of gradients designed to prepare membrane-bound polysomes, Dr. M. Silverman for the gift of antihook serum, Dr. K.O. Hedlund for advice about electron microscopy, and Drs. Randall and S.J.S. Hardy for comments on the manuscript. This work was done while the authors were at the Wallenberg Laboratory and the Molecular Biology Institute, University of Uppsala, Uppsala, Sweden. It was supported in part by grants from the United States Public Health Service and the Swedish Natural Sciences Research Council.

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