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Chemotaxis in *Bacillus subtilis*: Effects of Attractants on the Level of Methylation of Methyl-Accepting Chemotaxis Proteins and the Role of Demethylation in the Adaptation Process†

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ABSTRACT: By performing in vivo methylation experiments and using highly resolving $NaDodSO_4$–polyacrylamide gels, we have examined the effects of amino acid attractants on the methylation profile of *Bacillus subtilis* MCPs. Both increases and decreases have been found to occur in the level of methylation of these proteins. By using competition experiments and Conway diffusion cells, we have found that the demethylation event is correlated with the adaptation process. Gas chromatographic analysis indicates that methanol is evolved upon demethylation of these proteins. As more attractant receptors are titrated, corresponding increases in methanol evolution result. During this period of increased rate of methanol production, bacteria swim smoothly.

Bacterial chemotaxis is a primitive sensory system. It is the process by which bacteria sense chemicals in their environment and respond to them by migrating up attractant gradients or down repellent gradients. Bacteria in isotropic medium alternately swim smoothly and tumble. Swimming is correlated with counterclockwise rotation of the flagella (as viewed down the flagellum toward the cell body), and tumbling is correlated with clockwise rotation of the flagella (Macnab, 1978; Silverman & Simon, 1974). When a chemical attractant is added to a suspension of bacteria, one observes an increase in the length of time that the bacteria swim (Berg & Tedesco, 1974). The opposite occurs upon addition of repellents. After this initial response, the bacteria resume their prestimulus behavior even though the chemical stimulus is still present. This phenomenon is referred to as adaptation. In the Gram-negative bacterium *Escherichia coli*, the adaptation process has been correlated with the methylation of certain intrinsic membrane proteins known as methyl-accepting chemotaxis

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proteins (MCPs) (Goy et al., 1977). When mutants which lack specific MCPs are used, it has been shown that many attractants and repellents can be classified into groups based upon which MCP mediates their effects (Springer et al., 1977). The Gram-positive *Bacillus subtilis* also shows chemotaxis toward various chemicals, and a role for protein methylation has been implicated (Ullah & Ordal, 1981). The present study documents the changes that take place in methylation of MCPs elicited by various amino acid attractants and relates these changes to the adaptation process in *B. subtilis*.

Materials and Methods

Chemicals. L-[*methyl*-^{3}H]Methionine (78–93 Ci/mM) was obtained from Amersham Corp. L-Amino acids were used. All chemicals were reagent grade.

Bacterial Strains. OI 1085 is a chemotactically wild-type strain of *B. subtilis*, which is *trpF*$_7$ *hisH* (formerly *hisB*) *metC*. Strain OI 1100 was obtained from OI 1085 by mutagenesis and is a chemotaxis mutant (Ullah & Ordal, 1981).

In Vivo Methylation. In vivo methylation experiments were performed on bacteria as previously described (Ullah & Ordal, 1981), except that bacteria were grown from spores rather than from a frozen culture. Protoplast incubation buffer was used with the following modifications: 25 mM potassium phosphate, 20 mM magnesium chloride, and 100 μg of chloramphenicol per mL. In vivo methylation experiments were initiated by adding 20 μL of [^{3}H]methionine to a 2.5-mL bacterial suspension followed by a 1-min incubation. An amino acid in protoplast buffer (pH 7) was then added, to achieve the desired concentration, and the suspension was incubated for an additional 1–5 min. The reaction was terminated by immersing the flask of cells into a dry ice/acetone bath. The culture was thawed at 4 °C and centrifuged to pellet the protoplasts, and the pellets were dissolved in 50 μL of Laemmli sample buffer (Laemmli, 1970). Samples were boiled for several minutes and applied to NaDodSO$_4$[1]–polyacrylamide gels [10% acrylamide and 0.125% bis(acrylamide)] (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue R, destained, and scanned for protein. The gels were then prepared for fluorography by the method of Laskey & Mills (1975). Fluorograms were scanned (as were the stained gels) by using an Ortec Model 4310 densitometer. Quantitation was performed by measuring peak areas with a Keuffel and Esser planimeter and dividing the labeled protein area by the stained protein area.

Tethering Experiments. Tethering experiments were performed as previously described (Goldman & Ordal, 1981). Briefly, bacteria were sheared to remove most flagella and attached to a coverslip via antiflagellar antibody. The coverslip was inverted onto a slide and supported by two flanking coverslips, creating a chamber. Solutions were passed through the chamber and the rotating bacteria analyzed by videotaping through a microscope. "Competition/tethering" experiments, based on "jamming capillary assays" (Ordal et al., 1977), were carried out by introducing the amino acid "competitor" to the tethered cells. After adaptation occurred, the competitor (at the same concentration as originally applied) and a second amino acid, the "attractant", were added together, and the adaptation time was determined.

Conway Diffusion Cell Analysis of Volatile Radioactive Product. For determination of the amount of volatile radioactive product produced by the bacteria, a method based on that used in analysis of *E. coli* volatile products was employed (Toews & Adler, 1979). Bacteria were grown and washed as described for in vivo methylations except that chemotaxis buffer was substituted for protoplast buffer, lysozyme was omitted, and chloramphenicol was present at 100 μg/mL. A 5-mL suspension of bacteria was methylated with 40 μL of [^{3}H]methionine for 5 min. The cells were then collected on 0.45-μm membrane filters (Millipore HAWP type) and washed twice with chemotaxis buffer containing chloramphenicol. The cells were resuspended (at the same concentration used during the methylation reaction) in chemotaxis buffer containing chloramphenicol; 200 μL of these cells was transferred to the outer well of a Conway diffusion cell, which contained 20 μL of formaldehyde opposite the bacteria. Amino acid attractant was then added (usually 20 μL of a 10-fold concentrated stock) to the bacteria, and the Conway cells were closed. The Conway cells were incubated for various lengths of time, and the reaction was stopped by rotating the Conway cell so the bacteria and the formaldehyde were mixed. The Conway cells were allowed to sit for another 4 h, enabling radioactive volatile product to equilibrate between the outer and center wells (center well containing 0.5 mL of water). At this time the Conway cell was opened, and an aliquot was removed from the center well and analyzed for radioactive product by liquid scintillation spectroscopy. Competition experiments were performed in Conway cells by preincubating the [^{3}H]methionine-labeled bacteria in the Conway cell with the amino acid competitor long enough for the bacteria to adapt to the competitor. The amino acid attractant was added, and the Conway cells were closed and treated as above.

Gas Chromatography. Volatile ^{3}H-labeled products were analyzed by a method similar to that of Toews and Adler (Toews & Adler, 1979). Gas chromatography was performed by using a Varian Aerograph series 1520 equipped with a Porapak Q column. Helium served as the carrier gas at a flow rate of 60 mL/min. The temperatures of the injector, column, and detector were 250, 135, and 215 °C, respectively. Immediately after sample injection, timed fractions were collected by bubbling the column eluate through 5 mL of Supersol scintillation fluid in vials, and the volatile radioactive products were measured by liquid scintillation spectroscopy.

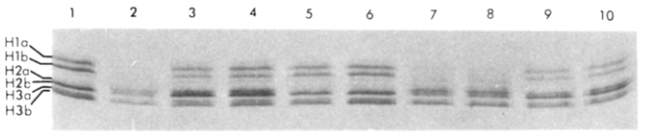

FIGURE 1: Effects of amino acids on methylation profile of *B. subtilis* MCPs. In vivo methylation experiments were performed as described under Materials and Methods. Amino acids were added at 10 times the K_d concentration (Ordal et al., 1977). (Lane 1) Buffer control; (lanes 2, 7, and 8) aspartate; (lanes 3 and 4) histidine; (lanes 5 and 6) cysteine; (lanes 9 and 10) glutamate.

Results

Effects of Amino Acid Attractants on Methylation of MCPs. Bacteria were exposed to 10 times the K_d concentration of amino acid[2] (Ordal et al., 1977) in the presence of [^{3}H]-methionine, and the proteins were analyzed on 10% polyacrylamide gels (Laemmli, 1970). Labeled proteins were

[1] Abbreviation: NaDodSO$_4$, sodium dodecyl sulfate.

[2] Throughout this paper we use the terms K_d, binding site, and receptor occupancy. Although based on chemotaxis experiments, these are valid terms, assuming the amino acid ligand is in rapid equilibrium with its receptor. This has been shown to be the case for galactose and ribose and their respective binding proteins, where the K_d's calculated from chemotaxis experiments are in agreement with those calculated from in vitro binding experiments (Mesibov et al., 1973; Zukin et al., 1977; Spudich & Koshland, 1975).

Table I: Effects[a] of Amino Acids on MCP Methylation

amino acid[b]	protein band[c]					
	H1a	H1b	H2a	H2b	H3a	H3b
Ala	NC	–	NC	NC	NC	NC
Arg	NC	–	NC	NC	NC	–
Asn	–	–	+++	NC	–	–
Asp	–––	–––	+++	–	–	–
Cys	NC	–	NC	NC	NC	NC
Gln	–	––	+++	+	–	–
Glu	––	––	+++	NC	–	–
Gly	–	–	NC	+	NC	+
His	–	––	+++	++	–	–
Ile	–	–	NC	NC	NC	–
Leu	–	–	NC	NC	NC	–
Lys	–	––	NC	–	–	–
Phe	NC	––	NC	NC	–	–
Pro	–	–	NC	+	NC	NC
Ser	–	–	NC	+	–	–
Thr	NC	–	NC	NC	–	–
Val	NC	–	NC	+	+	NC

[a] Effects were tabulated as follows: +, ++, and +++ represent 10–30%, 30–60%, and 60% or greater increases in methylation, respectively. –, ––, and ––– represent 10–30%, 30–60%, and 60% or greater decreases in methylation, respectively. NC signifies no detectable change in methylation level. Data were obtained from fluorograms of in vivo methylation experiments. [b] Amino acids were used at a final conentration of $10K_d$ (Ordal et al., 1977). [c] Protein bands refer to those illustrated in Figure 1.

visualized by fluorography (Laskey & Mills, 1975). These gels, which contained a lower bis(acrylamide) to acrylamide ratio than previously used (Ullah & Ordal, 1981), allowed resolution of *B. subtilis* MCPs into five or six major bands which were designated H1a through H3b (Figure 1). Amino acids were found to cause a variety of effects on the methylation of *B. subtilis* MCPs (Figure 1 and Table I). When the attractant histidine was added to the bacteria, a decrease in the level of methylation of bands H1a and H1b was observed, with H1b being preferentially demethylated. Histidine was also observed to stimulate the methylation of bands H2a and H2b. The effects of aspartate were more dramatic. This amino acid at 10 times its K_d concentration resulted in almost complete demethylation of bands H1a and H1b and increased methylation of H2a. Only asparagine, aspartate, glutamate, glutamine, and histidine caused an increase in the methylation of band H2a. These amino acids all caused some demethylation of bands H1a and H1b and slight demethylation of bands H3a and H3b. The effects of the other amino acids on the levels of MCP methylation were not as dramatic as those mentioned above. By careful quantitation, moderate effects were discerned. For these amino acids some demethylation of bands H1a and/or H1b occurred upon addition at 10 times the K_d concentrations. Protein band H2a was not affected by these amino acids, and bands H2b, H3a, and H3b had either slight increases, decreases, or no effect on the level of methylation. The previous finding of Ullah & Ordal (1981) that isoleucine causes methylation, rather than demethylation, of MCPs is incorrect (see Table I) due mainly to poor resolution of proteins in these gels (high ratio of bis(acrylamide) to acrylamide) and to the failure to normalize for amount of protein applied.

Which of these multiple effects is related to chemotaxis in *B. subtilis*? By varying the concentration of a particular amino acid, which produces a corresponding change in the response of the bacteria (Goldman & Ordal, 1981; Ordal et al., 1977), one can look for analogous changes in the methylation patterns of the MCPs. This rationale was used to analyze the methylation profiles for the amino acids histidine and aspartate

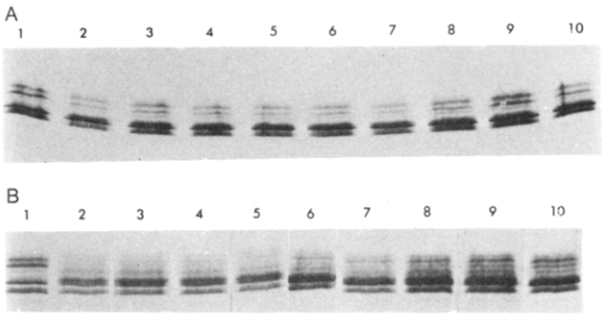

FIGURE 2: Effects of various concentrations of (A) histidine and (B) aspartate on the methylation of *B. subtilis* MCPs. The experimental protocol is described under Materials and Methods. In both (A) and (B), lane 1 corresponds to buffer control and lanes 2–10 correspond to adding amino acid at concentrations so 90–30% of its receptors are titrated: (lanes 2 and 3) 90%; (lanes 4 and 5) 80%; (lanes 6 and 7) 70%; (lanes 8 and 9) 50%; (lane 10) 30%. The experiment was performed in duplicate on the 90%, 80%, 70%, and 50% samples and quantitation of gels was routinely done as described under Materials and Methods.[3]

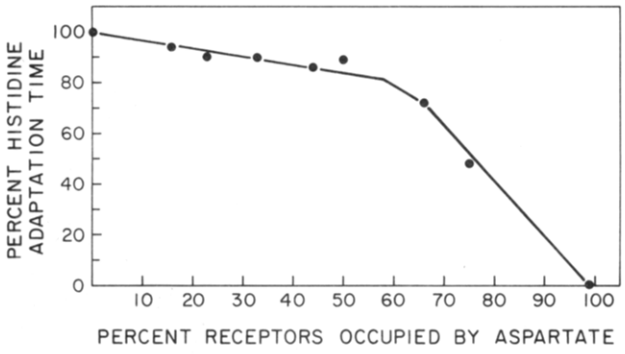

FIGURE 3: Effect of increasing aspartate concentration on the histidine adaptation time. The protocol for competition experiments on tethered cells is described under Materials and Methods. Aspartate was added to the bacteria at concentrations corresponding to 0–98% of its receptors titrated. After adaptation to aspartate was complete, aspartate (at the same concentration) and histidine (0.032 M, 90% receptor occupancy) were added. Adaptation time was determined and is reported as percent of the histidine adaptation time when no aspartate was present.

(Figure 2). In these experiments sufficient amino acid was added to titrate 0–90% of its apparent binding sites.[2] Direct observation and quantitation, as described under Materials and Methods, revealed that for histidine, as one titrated more of its receptors,[3] a progressive demethylation of band H1b and a progressive methylation of bands H2a and H2b took place. Thus either or both of these effects may be involved in mediating the behavioral response to this attractant. In the case of aspartate, the results were more clear-cut. As one increased the number of receptors occupied by aspartate, one observed a progressive decrease in the level of methylation of band H1a while bands H1b, H2a, and H2b remained fairly constant at all concentrations of aspartate tested.

Competition experiments on tethered bacteria were employed to determine how histidine's effects on MCP methylation are related to the behavioral response. The competitor was aspartate, and the attractant was histidine. Bacteria were first incubated in the competitor and allowed to adapt. After adaptation was complete, the competitor and the attractant

[3] Although the duplicate samples appear different in extent of methylation, this is an unnormalized gel, and as pointed out under Materials and Methods, these are quantitated and the difference vanishes. Thus, the differences observed between duplicates reflect different amounts of protein being applied to the gel.

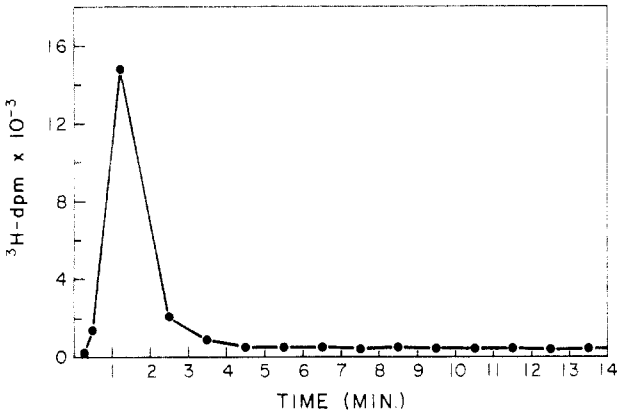

FIGURE 4: Gas chromatograph analysis of volatile radioactive product. Bacteria were exposed to aspartate, and volatile radioactive product was trapped and concentrated as described under Materials and Methods.

were added, and the adaptation time was determined (Figure 3). If bands H2a or H2b mediate the histidine response, then aspartate (which affects these bands to the same extent when 30–90% of its binding sites are titrated) should affect the histidine response equally at any concentration within this range. It was observed that the histidine response was slightly affected (10% decrease in adaptation time) by the competitor aspartate when 30% of the aspartate receptors was titrated. Not until one titrated at least 60% of these receptors did one observe a dramatic decrease in the histidine adaptation time. When histidine serves as the competitor (at 90% receptor occupancy) and aspartate the attractant (90% receptor occupancy), it was observed that the aspartate adaptation time was decreased by 10%.

Effects of Amino Acids on Methanol Production. Previous work in our laboratory has indicated that *B. subtilis* MCPs are modified by methylation of a γ-carboxyl group of a glutamic acid residue (unpublished results). Demethylation of these proteins is expected to yield methanol. For determination of the product of demethylation, a large Conway cell was employed. The cell was constructed so that a 25-mL volume of cells could be placed in the outer chamber and 50 mL of water was employed as a trap in the center well. Bacteria were labeled for 10 min with [^{3}H]methionine, washed to remove unreacted [^{3}H]methionine and placed in the outer chamber of the Conway cell. Aspartate was added to a final concentration of 10 times its K_d, and the Conway cell was closed. The reaction was terminated after 10 min, and after 10 h of equilibration the contents of the center well were distilled until approximately 1 mL was collected. The distillate was then analyzed by gas chromatography and liquid scintillation spectroscopy (Figure 4). Only one volatile radioactive peak was obtained, and this peak comigrated with authentic methanol (data not shown). The recovery of radioactivity from the gas chromatograph was 91%. Analysis of the chemotactic mutant OI 1100 (Ullah & Ordal, 1981), in which very little methylation of the MCPs takes place, gave a single radioactive peak with about 10% of the radioactivity of wild type, which also comigrated with authentic methanol.

The effects of various amino acid attractants on methanol production were determined with the use of Conway diffusion cells. Controls received buffer instead of attractant. After equilibration, an aliquot from the center well was collected and counted for radioactivity. The percent increase in methanol produced is presented in Table II. Of all amino acids tested, aspartate resulted in the greatest increase in methanol evolved.

Table II: Effects of Amino Acids on Methanol Evolution

amino acid[a]	increase in [^{3}H]methanol evolved[b] (%)
Asp	200
His	118
Ser	93
Gly	63
Pro	44

[a] Amino acids were used at a final concentration of $10K_d$ (Ordal et al., 1977). [b] Values are reported as percent above buffer control. Experiments were performed by using Conway diffusion cells.

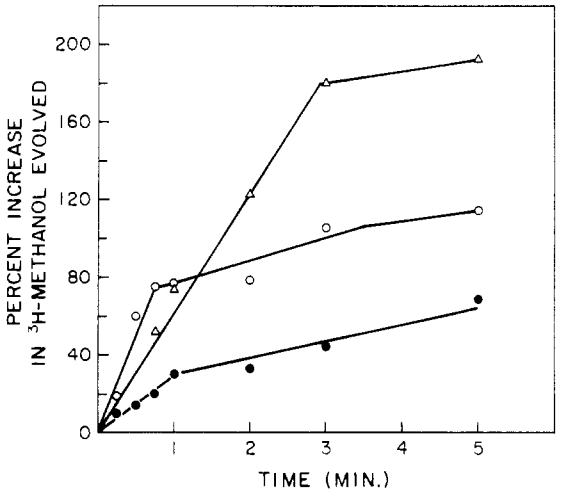

FIGURE 5: Time course of methanol evolution as a result of exposing bacteria to aspartate (△), histidine (○), or buffer (●). The concentration of amino acid was adjusted so as to titrate 90% of its receptors. Experiments used Conway diffusion cells as described under Materials and Methods.

Is methanol production related to the length of time it takes a bacterium to adapt to an amino acid attractant? For investigation of this, time course experiments were carried out by utilizing Conway diffusion cells. The reaction was allowed to proceed for various lengths of time. For 0.1 M aspartate (90% receptor occupancy), an increase in the rate of methanol production was observed for 3 min and was then followed by a rate similar to that of the control (Figure 5). Under the same conditions free-swimming bacteria adapted to 0.1 M aspartate in 3.5 min. Addition of histidine (0.032 M, 90% receptor occupancy) caused a 45-s enhancement of the rate of methanol production (Figure 5) and was followed by 45 s of smooth swimming.

If methanol production is related to the adaptation process, one would expect that as one titrates the receptors for an amino acid, a concomitant change in both methanol evolved and behavior would take place. Conway cells were employed to investigate this relationship. The data in Figure 6 show that as more receptors were titrated with the attractants aspartate, histidine, or serine, more methanol was evolved. In addition, methanol production was greatest with aspartate and least with serine.

Aspartate was effective at preventing the response to histidine (Figure 3), but histidine was relatively ineffective at even reducing the response to aspartate. Conway diffusion cells were also used to investigate the role methanol evolution may play in mediating these responses. Bacteria previously labeled with [^{3}H]methionine and washed were exposed to the competitor for 5–10 min followed by addition of the attractant and closing of the Conway cell. The reaction was allowed to continue for another 2–5 min, depending on the attractant's

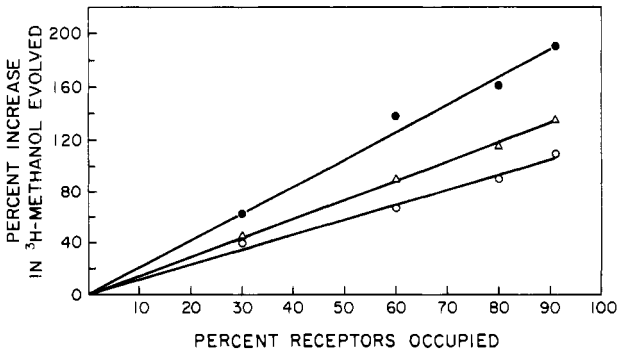

FIGURE 6: [^{3}H]Methanol evolution as a function of attractant concentration. [^{3}H]Methanol was determined by using Conway diffusion cells as described under Materials and Methods. Attractants, aspartate (●), histidine (△), and serine (○), were used at concentrations corresponding to 0–90% receptor occupancy.

adaptation time. After equilibration, an aliquot from the center well was counted for radioactivity. Preincubation with aspartate resulted in no further increase in methanol production upon subsequent addition of histidine. However, preincubation with histidine followed by exposure to aspartate did result in additional methanol production (80% increase).

Discussion

The experiments reported here were undertaken in order to determine the effects of amino acid attractants on the methylation of *B. subtilis* MCPs and the role these effects play in chemotactic behavior. We have found that both an increase and a decrease in the level of methylation of *B. subtilis* MCPs may occur in response to attractant stimulation. This is a unique finding in bacterial chemotaxis. Such effects have been most extensively studied in *E. coli* and *S. typhimurium*, both of which show an increase in the level of methylation of MCPs upon attractant stimulation (Goy et al., 1977; Paoni & Koshland, 1979).

For determination of which of these multiple effects may mediate the chemotactic response (Figure 2), in vivo methylation experiments were performed with aspartate and histidine over a range of concentrations which result in different adaptation times (Goldman & Ordal, 1981). These experiments indicated that the demethylation of protein band H1a and/or H1b was involved in mediating the adaptation to aspartate. If band H1b was involved in mediating the aspartate response, then the use of histidine as a competitor in competition experiments on tethered cells would be expected to decrease the aspartate adaptation time significantly, since histidine causes a substantial demethylation of this band (approximately 40–50%) when 90% of its receptors is occupied. It is observed that histidine, at a concentration equivalent to titrating 90% of its receptors, can decrease the aspartate adaptation time by at most 10%, which is in agreement with the amount of demethylation of band H1a. Band H1a, therefore, appears to be involved in mediating the aspartate response.

Histidine affected the amount of methylation in bands H1b, H2a, and H2b at concentrations that also affected bacterial behavior. "Competition" experiments on tethered bacteria were undertaken in order to elucidate which of these multiple effects were involved in mediating adaptation to histidine. Since aspartate stimulates methylation of bands H2a and H2b to the same extent at 30% receptor occupancy as at 90%, one can predict the effect different concentrations of aspartate would have on the histidine adaptation time. If protein bands H2a and/or H2b mediated the histidine response, then aspartate as competitor should be equally effective at reducing the histidine response when it is present at concentrations equal to 30% or 90% receptor occupancy. One obtains a significant decrease in the histidine adaptation time (Figure 3) only when the aspartate concentration is high enough to titrate about 60% of its receptors. Furthermore, the methylation profile of bacteria exposed to aspartate at 90% receptor occupancy compared to the methylation profile obtained by first exposing the bacteria to that concentration of aspartate followed by histidine (at 90% receptor occupancy) resulted in an increased stimulation of bands H2a and H2b due to the addition of histidine (unpublished observation). Tethered bacteria, however, showed no response to histidine under these conditions (Figure 3). This implies that bands H1a and/or H1b are involved in mediating the histidine response. On the basis of in vivo methylation experiments where histidine was varied over a range of concentrations resulting in titration of 30–90% of its receptors (Figure 2A), it appears that protein band H1b changed its methylation profile most consistently with the change in the number of receptors titrated.

Ideally, we would like to have mutants lacking specific MCPs to confirm which attractants mediate their effects through a particular MCP. Such mutants are currently being sought so that this goal can be achieved.

If histidine and aspartate were interacting at the same site, one would expect that when either was present as competitor (90% receptor occupancy) in the competition experiments on tethered cells, complete loss of the attractant's response would occur. Also, one would expect the same effect on the level of methylation of that particular protein if they shared a common site on the MCP. However, aspartate prevented histidine response virtually nonreciprocally and caused much greater demethylation of H1b. It is likely that histidine and other amino acids that cause less demethylation of H1b affect its methylation by binding at other receptors (Ordal et al., 1977).

The product of demethylation of the MCPs is expected to be methanol since these proteins are methylated on the γ-carboxyl group of a glutamic acid residue (unpublished results). Therefore, Conway diffusion cells were employed to measure the volatile radioactive product in response to attractant stimulation (Table II). Analysis by gas chromatography confirmed that all volatile radioactive product was methanol (Figure 3). The time course experiments (Figure 5) indicate that the increased rate of methanol production was correlated with the adaptation time of the bacteria. Aspartate, with an adaptation time of about 3.5 min, had an increased rate of methanol production for about the same length of time (3 min). A similar situation exists for histidine, which has an adaptation time of about 45 s. The slight increase in rate of methanol production by the control for the first minute may represent an oxygen effect due to addition of buffer.

If demethylation of the MCPs is involved in mediating the response to attractants, then it is expected that as one progressively titrates the attractant's receptor, a corresponding increase in methanol evolution would take place. We have previously shown that the adaptation time to amino acid attractants is proportional to the percent of receptors titrated by that amino acid (Goldman & Ordal, 1981). Here we show that the amount of methanol evolved is also proportional to the percent of receptors occupied by the amino acid attractant (Figure 6). Furthermore, the correlation between methanol production and stimulation of smooth swimming is underscored by histidine–aspartate competition experiments. Preincubation of the bacteria with aspartate prevented both an increase in evolution of methanol and smooth swimming caused by addition of histidine, but preincubation with histidine only slightly

reduced the increase in evolution of methanol and duration of smooth swimming caused by addition of aspartate.

The methylation profiles resulting from the addition of different amino acid attractants to the bacteria may also shed light on the "jamming capillary assays" performed on *B. subtilis* (Ordal et al., 1977). In those experiments it was found that aspartate and glutamate were able to compete with all the amino acids tested as "victims" in a capillary assay. It is interesting that these two amino acids had the most dramatic effects, compared to the other amino acids, on the methylation of *B. subtilis* MCPs.

Time course experiments in which the rate of methylation of the MCPs was measured indicate that there is no significant change upon addition of attractant (unpublished data). There may be changes in the rate of methylation of individual proteins, but inadequate resolution makes quantitation difficult.

If, as it appears, the attractants mediate their effects via demethylation of MCPs, then one might expect that there exists chemicals that would result in inhibition of methanol production and thus be repellents. We are currently examining repellents for *B. subtilis* to determine their effect on methanol production.

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Effect of Phospholipid Oxidation Products on Transbilayer Movement of Phospholipids in Single Lamellar Vesicles†

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ABSTRACT: Single lamellar phosphatidyl[*methyl*-^{2}H]choline vesicles were incubated with an excess of unlabeled phosphatidylcholine vesicles or phosphatidylcholine–cholesterol vesicles containing 8 mol % glucuronosyldiglyceride. Incubation of the two vesicle populations was performed in the presence or absence of a purified phosphatidylcholine exchange protein. The negatively charged glycolipid donor vesicles could be completely removed by column chromatography on DEAE-Sephacel. Following incubation with exchange protein and subsequent fractionation, the $-N(CD_3)_3$ phosphatidylcholine acceptor vesicles exhibited a 61–73% enrichment of the unlabeled phosphatidylcholine in the outer monolayer. Upon incubation in an air atmosphere, no appreciable transbilayer movement of the outer monolayer $-N(CH_3)_3$ phosphatidylcholine was observed for at least 5 days. Between days 5 and 7, however, extensive transbilayer movement occurred, leading to an outer monolayer/inner monolayer phosphatidylcholine ratio of 2.1 on day 7. In phosphatidylcholine–6 mol % cholesterol vesicles treated similarly, the outside/inside ratio of the unlabeled phospholipid was 6.7, suggesting a much smaller percentage of transbilayer movement. The loss of transbilayer asymmetry which occurred during a 36-h period after day 5 could be estimated at the upper limit, $t_{1/2} \sim 7.3$ h for phosphatidylcholine vesicles and $t_{1/2} \sim 53$ h for phosphatidylcholine–cholesterol vesicles. The actual rates for transbilayer movement, however, were likely more rapid. Transbilayer movement occurred at a time period when oxidized phospholipid breakdown products had reached critical levels.

Transbilayer and transmembrane movement of phospholipid has been examined in a variety of synthetic single lamellar vesicles and specific biological membranes. In single lamellar vesicles, transbilayer movement of phosphatidylcholine (Johnson et al., 1975; Rothman & Dawidowicz, 1975; Shaw et al., 1977), lysophosphatidylcholine (Van Den Besselaar et al., 1977; DeKruijff et al., 1977), and phosphatidylinositol (Low & Zilversmit, 1980) is generally slow, with half-times on the order of many days. An increase in the transbilayer rate of movement of phospholipid can occur if the acyl chain composition of the two monolayers comprising the bilayer is

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