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Rapid Attractant-Induced Changes in Methylation of Methyl-Accepting Chemotaxis Proteins in *Bacillus subtilis*†

Mark S. Thoelke, Helen M. Parker, Erik A. Ordal, and George W. Ordal*
Department of Biochemistry, School of Chemical Sciences and College of Medicine, University of Illinois, Urbana, Illinois 61801

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ABSTRACT: In *Bacillus subtilis*, addition of chemotactic attractant causes an immediate change in distribution of methyl groups on methyl-accepting chemotaxis proteins (MCPs), whereas in *Escherichia coli*, it causes changes that occur throughout the adaptation period. Thus, methylation changes in *B. subtilis* are probably related to excitation, not adaptation. If labeled cells are exposed to excess nonradioactive methionine, then attractant causes immediate 50% delabeling of the MCPs, suggesting that a flux of methyl groups through the MCPs occurs. Methanol is given off at a high rate during the adaptation period and probably reflects demethylation of some substance to bring about adaptation. The fact that many radioactive methyl groups are lost immediately from the MCPs but only slowly arise as methanol is consistent with the hypothesis that they are transferred from the MCPs to a carrier from which methanol arises. Demethylation of this carrier may cause adaptation.

Chemotaxis is a primitive sensory mechanism whereby bacteria are able to sense their environment and migrate toward favorable conditions. Chemoattractants cause a period of smooth swimming before the cells return to prestimulus behavior (Berg & Tedesco, 1974; Ordal, 1975). In *Escherichia coli*, it has been shown that during this period of swimming attractants cause a cessation of methanol production and a progressive increase in the level of methylation of the specific methyl-accepting chemotaxis proteins (MCPs) that act as the receptors (Springer et al., 1977; Toews et al., 1979). Conversely, repellents cause augmented methanol production and demethylation of the MCPs during the period of induced tumbling (Goy et al., 1977; Toews & Adler, 1979). It is generally accepted that methylation changes of MCPs are central to adaptation (Goy et al., 1977). In *Bacillus subtilis*, it has been shown that attractants cause methylation changes in various MCPs and cause increased methanol production

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(Goldman et al., 1982). In this paper we have sought to investigate the time courses of these effects. The redistribution of methyl groups on the MCPs occurred immediately upon addition of attractant and thus is probably connected with excitation. Methanol release, however, spanned the smooth swimming period and thus is probably connected with adaptation.

Experimental Procedures

Strains. OI1085 is a chemotactically wild strain of *B. subtilis*, which is *trpF7*, *hisH2*, and *metC* (Ullah & Ordal, 1981). OI1100 is OI1085 *cheR* and lacks chemotactic methyltransferase (Ullah & Ordal, 1981).

Chemicals. L-[*methyl*-^{3}H]Methionine (75–80 Ci/mmol) was obtained from Amersham Corp. Electrophoresis reagents were all of electrophoresis grade. All other chemicals were of reagent grade.

Solutions and Media. Tryptone broth (TBr) is 1% tryptone and 0.5% NaCl. Luria broth (LBr) is TBr with 0.5% yeast extract added. Chemotaxis buffer (CB) and protoplast buffer (PB) are as described previously (Ullah & Ordal, 1981; Ordal & Goldman, 1975).

In Vivo Methylations. In vivo methylations were performed as described by Ullah and Ordal (1981). Washed cells were resuspended in PB at A_{600} = 1.0, with the addition of 1 mg/mL lysozyme and 0.1 mg/mL chloramphenicol. Methylation was initiated by the addition of 10 μCi/mL [*methyl*-^{3}H]-methionine. Reactions were performed at 25 °C. Effectors were added after 10 min of methylation unless specified otherwise. Samples were removed at various times before and after effector addition and frozen in a dry ice–acetone bath. The samples were thawed at 4 °C and the protoplasts pelleted by centrifugation. For cold chase conditions, nonradioactive methionine was added in excess (10^{-5} M final concentration).

For methanol assays, cells were resuspended in CB with chloramphenicol (0.1 mg/mL). Cells were labeled for 10 min at 25 °C with 30 μCi/mL [^{3}H]methionine.

In order to achieve constant specific activity of methyl groups, cells were grown up in LBr containing 50 μCi/mL [^{3}H]methionine and 10^{-4} M nonradioactive methionine. These cells were grown up to 180 Klett units and resuspended in CB.

SDS–Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (1970). After addition of Laemmli sample buffer, the samples were boiled for 7 min and fractionated on SDS–PAGE [10% acrylamide and 0.125% bis(acrylamide)]. Gels were prepared for fluorography by the method of Laskey and Mills (1975). Densitometer scanning was performed on an LKB Ultrascan LX.

Continuous-Flow Assay for Methanol Evolution. An assay based on that described by Khery et al. (1984) has been described previously (Thoelke et al., 1987). Labeled cells were loaded onto a 0.45-μM Millipore filter unit and had buffer pumped past them at a constant rate. The buffer contained excess nonradioactive methionine (10^{-5} M). When the buffer reservoirs were changed, the cells experienced addition and removal of effectors. The buffer was fractionated and assayed for volatile labeled product.

Cells labeled by growth in low specific activity [^{3}H]-methionine were loaded onto the filter after washing. In these assays, the buffer did not contain nonradioactive methionine. Any internal proteolysis supplying methionine would provide the same specific activity.

Time Course of Methanol Evolution. Cells were labeled for 10 min as described above and then filtered and washed with CB to remove any unincorporated [^{3}H]methionine. Cells were then resuspended in CB containing excess nonradioactive methionine (10^{-5} M). The reaction was incubated at 25 °C with gentle shaking to aerate the cells. Samples were removed periodically and placed in test tubes on ice containing formaldehyde to halt all reactions. Samples were assayed for labeled volatile product in the same manner as for the flow assay.

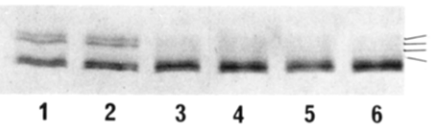

FIGURE 1: Time course of attractant-induced MCP methylation changes. Experiments were performed as described under Experimental Procedures. Cells were incubated in radioactive methionine at 25 °C. Aspartate was added at 10 min to a final concentration of 0.1 M (90% receptor occupancy). Samples were removed at times before and after addition. Lanes 1 and 2, 30 and 15 s prior to addition, respectively; lanes 3–6, 5, 15, 30, and 60 s after aspartate addition, respectively. Lines indicate positions of MCPs H1a, H1b, H1c, and H1d (top to bottom).

Results

Effect of Attractant on MCP Changes. All of the amino acids act as chemoattractants for *B. subtilis* (Ordal & Gibson, 1977). Upon exposure to amino acids, the bacteria increase the rate of methanol formation, change the distribution of methyl groups on the MCPs, and swim smoothly for a period of time befor adaptation occurs (Goldman et al., 1982; Goldman & Ordal, 1981). Of all the amino acids, aspartate causes the longest adaptation time (Goldman & Ordal, 1981). We therefore chose aspartate to observe the time course of the attractant-induced changes in the methylation profile of the MCPs. It has been shown previously that aspartate affects the level of methylation of more than one MCP (Goldman et al., 1982), in contrast to *E. coli*, where the change is seen in only the MCP that acts as the receptor (Springer et al., 1977).

Cells were radiolabeled in vivo with [^{3}H]methionine at 25 °C rather than at 37 °C in order to slow methyl-transfer reactions. At various times, aliquots were removed from the reaction mixture and frozen in a dry ice–acetone bath. The distribution of methyl groups changed within 5 s of addition of 0.1 M aspartate (90% receptor occupancy) and then remained fairly fixed (Figure 1). Aspartate caused demethylation of the upper bands (H1a, H1b), methylation of a lower band (H2), and genesis of intermediate bands. By comparison with Figure 1 of Goldman et al. (1982), these may be designated H1c and H1d. They appear to be more highly methyl-esterified forms of H1 (Goldman & Ordal, 1984). It has been shown that H1, H2, and H3 are distinct proteins (Bedale et al., 1988). Different concentrations of aspartate yield distinct distribution of methyl groups of the MCPs (Goldman et al., 1982). However, a densitometer scan of the fluorogram showed that the extent of labeling of the entire MCP region as a whole was unaffected by the attractant (Figure 2a). At later time points, an increase in the labeling of all MCPs occurred through basal turnover of methyl groups (Figure 1).

These changes in the methylation pattern on the MCPs occurred within 5 s of attractant addition. The rapid change in profile preceded the adaptation to the attractant by approximately 60 s. This is in contrast to the gradual methylation changes that are seen in *E. coli* (Goy et al., 1977).

The above experiment was also performed in "cold chase" conditions, by adding excess nonradioactive methionine 3 min before adding aspartate. Aspartate caused similar changes in the degree of methylation of each MCP, with the major difference being an overall loss of label in all of the MCPs of

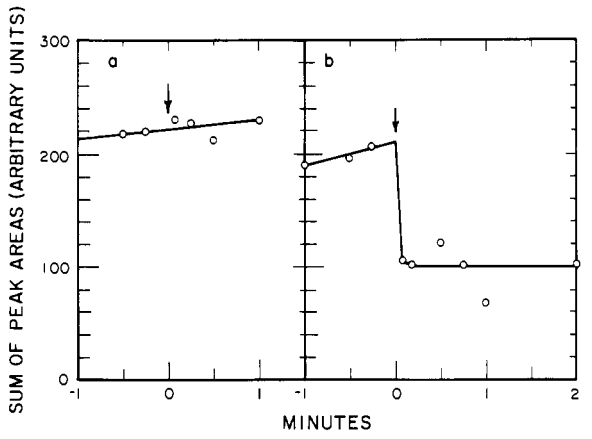

FIGURE 2: Densitometer scans of MCP regions. (a) Fluorogram from Figure 1. (b) Similar experiment but excess nonradioactive methionine added 2 min before aspartate. Aspartate added at $t = 0$ (arrow).

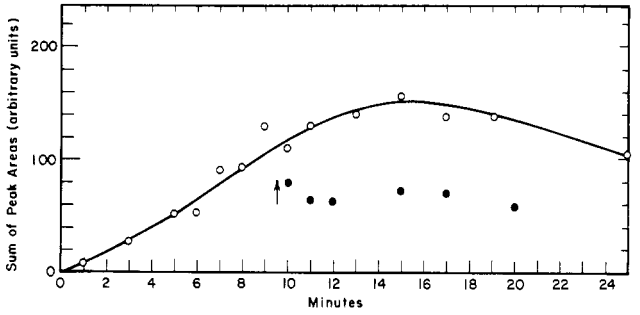

FIGURE 3: Time course of MCP labeling. Cells were labeled as described under Experimental Procedures. Aliquots were removed as a function of time before and after addition of excess nonradioactive methionine. These aliquots were either immediately frozen (O) or added to aspartate (0.1 M final concentration), mixed for 5 s, and then frozen (●). Samples were run on SDS–PAGE, and the resulting fluorogram was scanned on a densitometer.

approximately 50% (Figures 2b and 3). Once again all changes occurred within 5 s of aspartate addition and thereafter remained fairly constant. Therefore, attractant induces a flux of methyl groups through the MCPs, which is apparent when nonradioactive groups replace labeled groups (or vice versa) (Bedale et al., 1988; Thoelke, Casper, and Ordal, unpublished results).

Basal Rate of Turnover on MCPs. To determine the basal rate of turnover of methyl groups on MCPs at 25 °C, cells were labeled for 8 min before being given excess nonradioactive methionine, and aliquots were taken over time. These aliquots were either frozen directly or first exposed to aspartate (0.1 M) for 5 s and then frozen. The loss of label in unexposed samples was not immediate at 25 °C, indicating that the methionine uptake and activation to *S*-adenosylmethionine (Adomet) took some time (Figure 3). After a few minutes, the MCPs began to lose labeled methyl groups. Aspartate addition appears to have hastened this process many fold.

Effect of Attractant on Methanol Production. It has been shown that the only labeled volatile product released by [^{3}H]methionine-treated *B. subtilis* is methanol (Goldman et al., 1982). The methyltransferase mutant OI1100 has been shown to release only 10% the amount of methanol as wild type (Goldman et al., 1982). If the methanol released were the direct product of demethylation of the MCPs caused by the rapid profile changes and turnover, then one would expect a short burst of radioactive methanol production upon addition of aspartate in a cold chase experiment. Two assays were used to monitor radioactive volatile product. In the first, buffer was pumped past prelabeled cells into a fraction collector.

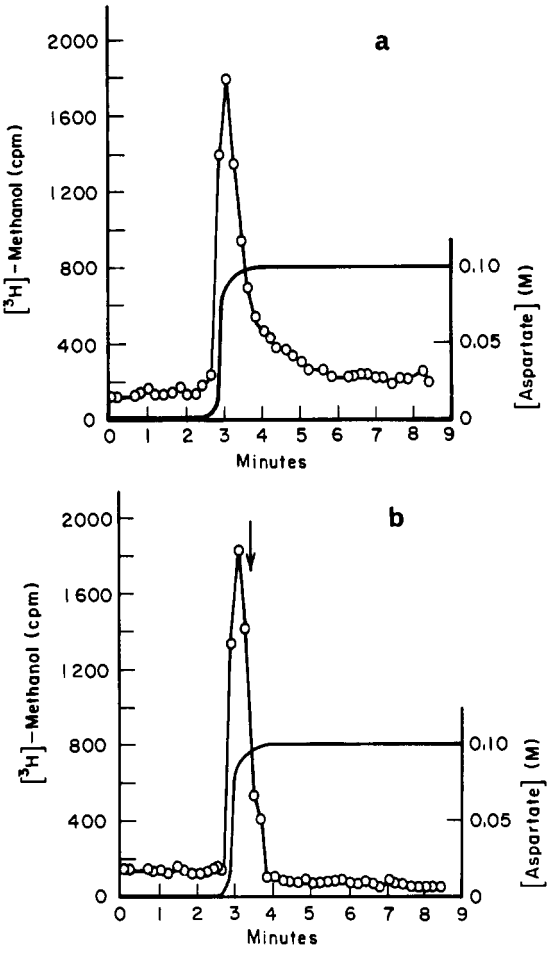

FIGURE 4: Effect of formaldehyde on aspartate-induced methanol production. Flow assay for methanol production performed as described under Experimental Procedures. Cells in (a) were exposed to 0.1 M aspartate. Cells in (b) were exposed to 0.1 M aspartate for 24 s before formaldehyde (1% final concentration) was added to the buffer reservoir (arrow). Solid line indicates aspartate concentration (determined by conductivity).

After sufficient flushing of accumulated waste products (approximately 1 min, data not shown), a flat base line of radioactive methanol production was attained (Figure 4a). This period of time in chase conditions, as shown above, would not cause a drop in the extent of MCP labeling. This flat base line, therefore, reflected the slow basal turnover of methyl groups on the MCPs at 25 °C. Upon switching the intake tube to buffer containing aspartate, there was an increase in labeled methanol which corresponded to rising aspartate concentrations. The rate of formation peaked at approximately 24 s after initial exposure and then slowly returned toward the prestimulus base line. Since some mixing of the solutions occurred in the tubing, the cells were exposed to 0.1 M aspartate after approximately 1 min, not at once. Ninety percent of the stimulus (85% receptor occupancy) should have been delivered by 24 s, and adaptation should have occurred by 1 min. It is interesting to note that even after 10 min of aspartate exposure the rate was not entirely back to base line (data not shown).

This prolonged methanol evolution could have arisen from continued production or from a short rapid period of production followed by a slower diffusion out of the cell. The latter possibility was tested for by two methods. In the flow assay, after a short exposure to aspartate, formaldehyde was added to the aspartate-containing buffer, killing the cells. The rate of methanol production rapidly fell as formaldehyde

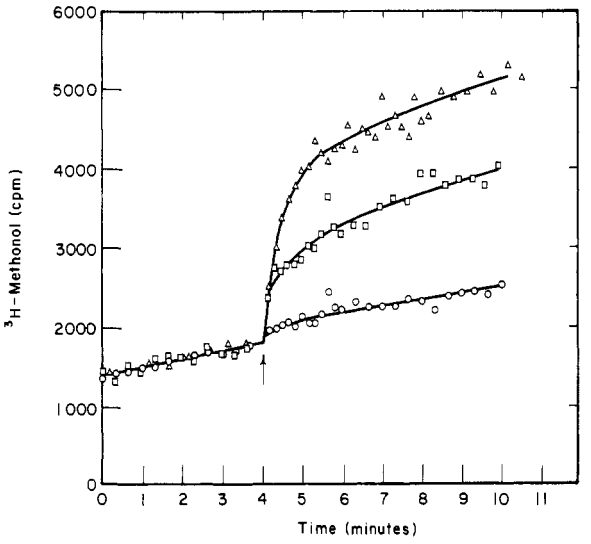

FIGURE 5: Time course of aspartate-induced methanol production. Experiment performed as described under Experimental Procedures. Labeled cells were given excess nonradioactive methionine and incubated at 25 °C. Aliquots were removed at various times, killed, and later assayed for labeled volatile product. Aspartate was added at 4 min (arrow). Experiments were performed on different days, and data obtained were different in respect to background and basal rate of methanol production. Therefore data are presented as relative amount of cpms, so that the slope of basal rate could be aligned. Symbols: (△) 0.1 M aspartate (90% receptor occupancy); (□) 1.0×10^{-2} M (50% receptor occupancy); (○) 4.3×10^{-3} M (30% receptor occupancy).

stopped the cellular reactions (Figure 4b). Formaldehyde would not have prevented diffusion of formed methanol. Therefore the continued detection of methanol was the result of continued formation. This conclusion was also reached by exposing the cells to a short burst of aspartate in a flow assay. If the methanol had all been generated upon initial exposure, then the subsequent removal of aspartate would not have affected the proposed slow release of methanol. It has been seen that, in fact, the methanol evolution rapidly falls upon removal of the aspartate (Thoelke et al., 1987).

In any case, the second assay circumvented the mixing problem by simply adding the aspartate directly into a flask containing the cold chase reaction mixture. Samples were removed over time from the mixture, killed, and then assayed. Assaying the whole reaction mixture yielded the same results as the supernatant with cells spun out (data not shown). It was seen that direct addition of aspartate caused immediate increase in methanol formation (Figure 5). The rate seemed similar for varying amounts of aspartate, and seemed to last for varying amounts of time, in direct relationship with concentration. The rate returned toward prestimulus base line at approximately the time of adaptation to the stimulus for 50% and 90% receptor occupancies of aspartate. However, 30% receptor occupancy caused only a few seconds of rate increase, significantly shorter than the time of induced smooth swimming expected. This discrepancy is currently being explored. After adaptation the rate was again slightly higher than the basal rate at higher concentrations of aspartate.

Evolution of Constant Specific Activity Methanol. To see whether the prolonged rate was due to increased amount of methanol or to increased specific activity, cells were grown for many generations in low specific activity [^{3}H]methionine. In this manner, all methionine incorporated into protein and all activated to Adomet was of the same specific activity. Therefore, all methyl groups transferred onto the MCPs and all methanol ultimately released by demethylation of the MCPs

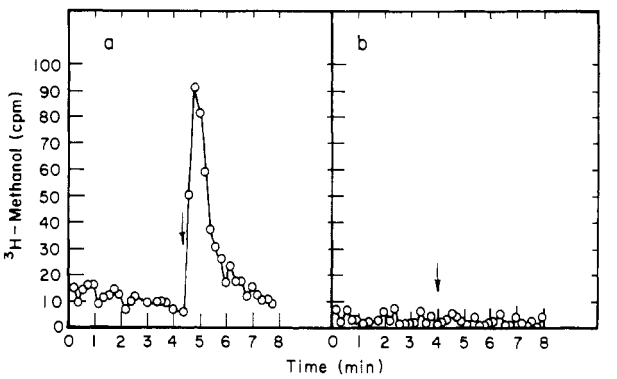

FIGURE 6: Methanol assays on cells labeled with fixed specific activity. Cells were grown in LBr containing low specific activity methionine (see text). A continuous-flow assay was performed as described under Experimental Procedures except that no methionine was added to the reservoirs. Cells in panel a were OI1085 (wild type), and cells in panel b were OI1100 and lack the chemotactic methyltransferase.

were also of this specific activity. These cells were exposed to aspartate in a continuous-flow assay in which no nonradioactive methionine was added to the buffer (Figure 6a). The increase in cpm's over background continued for at least 3 min after aspartate exposure. Thus, the prolonged response was at least in part due to an increase in the amount of methanol formed, not just a change in the specific activity.

The same assay was performed on the chemotactic mutant OI1100, which lacked the methyltransferase enzyme (Figure 6b). Here the basal rate of methanol production was lower than the wild-type strain, and aspartate had very little effect on methanol evolution. These findings confirm that the great majority of methanol produced is related to chemotaxis. The poor chemotaxis of OI1100 to attractants is evidence of the importance of methyl transfer in chemotaxis of *B. subtilis* (Ullah & Ordal, 1981).

Discussion

We have presented evidence for a disparity between the time courses of attractant-induced MCP profile changes and methanol formation in *B. subtilis*. Whereas distribution of methyl groups on MCPs changes immediately upon aspartate addition, methanol evolution continues at an increased rate and continues over a period of time corresponding to the period of induced smooth swimming. Therefore, the rapid changes on MCPs cannot be causing adaptation and would appear to be involved in excitation.

In *B. subtilis*, the only volatile labeled product released as a result of feeding [^{3}H]methionine is methanol (Goldman et al., 1982). An increased rate of methanol production due to attractant addition lasts for a relatively short period of time. In the continuous-flow assay the methanol evolution rate rapidly peaks and then slowly decays toward the base line. The removal of aspartate and the addition of formaldehyde both cause the acceleration of this return. This implies that the methanol is being formed more or less throughout this period of time above the base-line rate, not just during the first few seconds.

As is indicated by the aspartate concentration in Figure 4, there is a progressive increase of the attractant concentration due to mixing in the tubes leading to the cells, and from the cells to the fraction collector. This may have an effect on the time course of methanol release. We therefore used a second assay in which the aspartate was added directly into a methylation mixture. In this case it is clear that, immediately upon addition, a maximal rate of formation is achieved and it is the same for various concentrations of aspartate. The

period of time at this rate appears to depend on the concentration and roughly corresponds to amount of time in smooth swimming. It is likely that methanol release is involved in the adaptation response.

The immediate attractant-induced MCP changes in *B. subtilis* are in contrast to the reported changes in *E. coli*, which occur over the course of adaptation to the attractant (Goy et al., 1977). *B. subtilis* clearly remains swimming smoothly long after the MCP profile has been altered. Very little change in the overall extent of labeling of MCPs is seen upon attractant addition in *B. subtilis* is this experiment (Figure 2a). An immediate, overall loss of approximately 50% of label occurs when cells are incubated in excess nonradioactive methionine before being exposed to aspartate. This indicates a flux of methyl groups through the MCPs.

We have previously proposed that there exists an intermediate methyl acceptor to which groups may be transferred from the MCPs. This is supported by findings in this paper. It was shown that in cold chase conditions aspartate caused an immediate loss of labeled methyl groups on the MCPs. The extent of labeling thereafter remained fairly constant. These are the same conditions of cells in the methanol assays. It was shown that methanol is actively produced over time, not all at once, and slowly released. If the induced flux of methyl groups through the MCPs yielded methanol directly, the period of methanol evolution would last only a few seconds, not over a minute as shown in Figure 5.

In summary, we can contrast methyl group related events in *B. subtilis* and *E. coli*. In *B. subtilis*, MCP changes are immediate and may be related to excitation; in *E. coli*, MCP changes are gradual and occur over the period of adaptation (Goy et al., 1977). In *B. subtilis*, a rapid flux of methyl groups occurs upon excitation; in *E. coli*, no such flux occurs. In fact, even the normal turnover is halted during the adaptation period (Toews et al., 1979). In *B. subtilis*, methanol evolution occurs at a high rate during the adaptation period; in *E. coli*, methanol evolution stops (Toews et al., 1979). In *B. subtilis*, the weight of evidence suggests the existence of a new methyl group carrier to which methyl groups are transferred from MCPs; in *E. coli*, such a carrier is unknown. Indeed the chemotactic mechanisms, which must share a common origin since the MCPs are homologous (Burgess-Cassler & Ordal, 1982), have apparently diverged to an amazing extent.

Registry No. Methanol, 67-56-1.

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