Evidence for Methyl Group Transfer between the Methyl-Accepting Chemotaxis Proteins in *Bacillus subtilis*

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We present evidence for methyl (as methyl or methoxy) transfer from the methyl-accepting chemotaxis proteins H1 and possibly H3 of *Bacillus subtilis* to the methyl-accepting chemotaxis protein H2. This methyl transfer, which has been observed in vitro (D. J. Goldman and G. W. Ordal, Biochemistry 23:2600–2606, 1984), was strongly stimulated by the chemotactant aspartate and thus may play an important role in the sensory processing system of this organism. Although radiolabeling of H1 and H3 began at once after the addition of [3H]methionine, radiolabeling of H2 showed a lag. Furthermore, the addition of excess nonradioactive methionine caused immediate exponential delabeling of H1 and H3 while labeling of H2 continued to increase. Methylation of H2 required the chemotactic methyltransferase, probably to first methylate H1 and H3. Aspartate caused increased labeling of H2 and strongly decreased labeling of H1 and H3 after the addition of nonradioactive methionine. Without the addition of nonradioactive methionine, aspartate caused demethylation of H1 and to a lesser extent H3, with an approximately equal increase of methylation of H2.

Numerous biochemical processes are regulated by post-translational modification of proteins. In bacterial chemotaxis the reversible methylation of certain membrane-bound receptor proteins appears to play an important role in the adaptation of bacteria to chemical stimuli (8–10, 15). The specific functions these methyl-accepting chemotaxis proteins (MCPs) have in bacterial chemotaxis are not well understood, although changing levels of methylation of these proteins have been associated with the adaptation of bacteria to various attractants in *Escherichia coli* (5, 9, 13, 15). Recent work in this laboratory has focused on elucidating the pathway of methyl (as methyl or methoxy) group transfer from the MCPs to an intermediate carrier (or carriers) from which methanol arises during chemotaxis in *Bacillus subtilis* (16; M. Thoelke, J. Kirby, and G. Ordal, manuscript in preparation). One complication has been the possibility of transfer of methyl groups from one MCP to another. We recently noticed that the increase in methylation of one MCP (H2) seems to be approximately equal to the decrease in methylation of a different MCP (H1). The apparent shift in methylation from H1 to H2 is not merely due to a change in the degree of methylation of H1 resulting in a different mobility during electrophoresis, because H1, H2, and H3 are three distinct proteins (6; this study). In this report we present several lines of evidence that suggest that the methyl groups are transferred from H1 (and possibly H3) to H2.

**MATERIALS AND METHODS**

**Strains.** O11085 is a chemotactically wild-type strain of *B. subtilis* (17). O1100 is a chemotactic methyltransferase mutant (2, 17).

**Chemicals.** L-[methyl-3H]methionine (75 to 80 Ci/mmol) was obtained from Amersham Corp., New England Nuclear Corp., or ICN Pharmaceuticals Inc. Electrophoresis reagents were all electrophoresis grade. *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories, Inc. All other chemicals were reagent grade.

**Solutions and media.** Tryptone broth is 1% tryptone and 0.5% NaCl. Luria broth is tryptic broth with 0.5% yeast extract. Chemotaxis buffer and protoplast buffer were described previously (14, 17). *S. aureus* V8 protease solution is 0.5 μg of protease per ml in a buffer of 40 mM Tris hydrochloride, 0.2% sodium dodecyl sulfate (SDS), 2 mM EDTA, and 40% glycerol (pH 7.8) (4).

**In vivo methylations.** In vivo methylations were performed as described by Ullah and Ordal (17). Washed cells were suspended in protoplast buffer with 1 mg of lysozyme per ml. Methylation was initiated by the addition of [3H]methionine. In pulse-chase experiments the period of methylation was followed by the addition of a 100-fold excess of nonradioactive methionine for 2 min. Effector solutions were then added in some experiments for 30 s, at which time the reaction was terminated by freezing in a dry ice-acetone bath. The suspensions were thawed, and the protoplasts were pelleted at 4°C.

**SDS-PAGE.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (11). After the addition of 100 μl of Laemmli sample buffer, the samples were boiled for 7 min and fractionated by SDS-PAGE (10% acrylamide and 0.125% bisacrylamide unless otherwise indicated). Gels were prepared for fluorography by the method of Laskey and Mills (12). Fluorograms were scanned with a Zeineh soft-laser scanning densitometer or an Ortec model 4310 densitometer, and peak areas were integrated automatically or with a Keuffel and Esser polar compensating planimeter.

**Paper chromatography.** For paper chromatography, the method of Ahlgren and Ordal (1) was used. After centrifugation of bacteria methylated in vivo, 10 μl of the supernatant was spotted onto a 1-in. (2.54 cm)-wide strip of Whatman 3MM paper. The chromatogram was developed by descending paper chromatography in a solvent system of...
n-butanol-acetic acid-water (12:3:5 [vol/vol/vol]) for 14 h. The strips were then dried and cut into 1-cm slices, and the radioactivity was determined by liquid scintillation counting.

Limited proteolysis. For limited proteolysis, the method of Cleveland (4) was used, with some modifications. In vivo methylation was performed, and the samples were fractionated by standard SDS-PAGE as described above. The MCP bands H1, H2, and H3 were visualized by fluorography, excised from the dried gel with a razor, and rehydrated as described previously (4). The sample wells of a second SDS gel (15% acrylamide, 0.186% bisacrylamide; 2 mm thick) were loaded with 50 μl of S. aureus V8 protease solution, and current was applied to allow the sample to just enter the stacking gel. The power was turned off, the hydrated gel pieces were inserted into the sample wells, and current was applied again until the samples were 1 cm above the stacking gel-running gel interface. The power was turned off for 10 min to allow proteolysis to occur. The electrophoresis was then continued as usual, and the resultant gel was subjected to fluorography.

RESULTS

Limited proteolysis of MCPs. Multiple methylation of the same protein is believed to cause the fine banding pattern on fluorograms of SDS gels within each MCP region, such as H1α, H1a, H1b, and H1c within the H1 region (6), and MCPs in E. coli show similar patterns (3, 5). The MCPs in each of the three regions, H1, H2, and H3, are believed to be distinct (6). To definitively show that the individual MCPs are different proteins, limited enzymatic proteolysis of radiola-

beled H1, H2, and H3 was performed (Fig. 1). The pattern of peptide fragments produced clearly shows that H1, H2, and H3 are distinct proteins, as expected.

Time course of MCP methylation. To compare the individual rates of methylation of the MCPs H1, H2, and H3, cells were methylated as described in Materials and Methods. Samples were removed at various times and subjected to SDS-PAGE and fluorography. If methyl groups on H2 originate from H1 or H3 rather than directly from S-adenosylmethionine (SAM), there should be a lag in labeling H2 compared with H1 or H3, since at early times when radioactive methyl groups are first being transferred to H1 or H3, the groups being transferred from H1 or H3 to H2 will be nearly all nonradioactive. Moreover, if the radioactive methionine in the medium is exhausted and internal nonradioactive methionine from proteolysis becomes the source of methyl groups, then delabeling of H1 and H3 should precede delabeling of H2. Indeed, there was much slower initial methylation of H2 relative to H1 and H3, but after approximately 12 min the label incorporated in H1 and H3 began to decrease while the label in H2 continued to increase (Fig. 2). The methylation time courses for the individual MCPs were thus quite consistent with the idea of a methyl transfer from H1 (or H3 or both) to H2. Analysis of the medium after the initiation of methylation showed that, as expected, the

FIG. 1. Limited enzymatic proteolysis of MCPs. MCP regions H1, H2, and H3 were excised from an SDS gel and subjected to limited proteolysis (see Materials and Methods). The resulting peptide fragments of H1 (lane 1), H2 (lane 2), and H3 (lane 3) were separated by SDS-PAGE and fluorographed.

FIG. 2. In vivo time course of MCP methylation of B. subtilis O11085. (A) Strain O11085 was subjected to in vivo methylation, with methyllations terminated by freezing after 0.5 min (lane a), 1 min (lane b), 2 min (lane c), 4 min (lane d), 6 min (lane e), 8 min (lane f), 12 min (lane g), or 16 min (lane h). The resultant fluorogram is shown. (B) Densitometer scans of the individual MCP bands H1 (●), H2 (○), and H3 (□).
radioactive methionine disappeared rapidly (Fig. 3). Incidentally, this rapid loss of \[^3\text{H}\]methionine was not predominantly caused by methylation-demethylation reactions related to chemotaxis since it occurred as rapidly in a strain lacking the chemotactic methyltransferase responsible for MCP methylation (Fig. 3).

**Pulse-chase experiments.** No MCPs were labeled in a strain lacking the chemotactic methyltransferase (17); therefore, all methyl groups on H2 must arise from SAM via that enzyme, at least indirectly. The requirement for the chemotactic methyltransferase in the methylation of H2 has also been confirmed in vitro (6). More definitive experiments showing that label at H2 arose from H1 or H3 were performed by adding excess nonradioactive methionine after a brief incubation in \[^3\text{H}\]methionine. Again, labeling of H2 continued to increase even after labeling of H1 and H3 started to decrease (Fig. 4). H1 and H3 are the best candidates for the source of methyl groups at H2 since labeling of H2 required the chemotactic methyltransferase, as described above, and no other peptides were sufficiently labeled to be the source of the \[^3\text{H}\]methyl groups. Methyl transfer from H1 or H3 to H2 thus appears likely.

**Attractant stimulation of methyl transfer.** To determine whether this transfer of methyl groups is likely to play a role in chemotaxis, cells were labeled with \[^3\text{H}\]methionine and then given excess nonradioactive methionine for 2 min followed by the addition of attractant for 30 s. The amount of radioactivity associated with H2 increased much more after the addition of attractant than after the addition of the buffer control (Fig. 5 and Table 1). Since the SAM synthesized inside the cells was essentially nonradioactive, the new \[^3\text{H}\]methyl groups at H2 must have arisen from the other MCPs.

**DISCUSSION**

In this report evidence is presented for the existence of methyl transfer from MCP H1 and possibly H3 to MCP H2; this transfer was stimulated by the chemotaxis attractant aspartate. First, \[^3\text{H}\]methyl labeling of H2 had a lag, whereas labeling of H1 and H3 did not; furthermore, H2 \[^3\text{H}\]methyl labeling levels continued to increase even after \(^3\text{H}\) methylation levels at H1 had begun to decrease. Second, in a pulse-chase experiment H2 became increasingly labeled even after the addition of excess nonradioactive methionine but H1 and H3
FIG. 5. MCP methylation changes in response to aspartate. In vivo methylation was performed as described in Materials and Methods. (A) Cells were treated with protoplast buffer (lane 1) or 23 mM aspartate (lane 2) for 30 s before being frozen in a dry ice-acetone bath. (B) Cells were given a 100-fold excess of nonradioactive methionine after methylation and incubated for 2 min before the 30-s treatment with protoplast buffer (lane 1) or 23 mM aspartate (lane 2).

did not. The source of the new methyl groups at H2 must be a substance methylated at least indirectly by the chemotactic methyltransferase, presumably H1 and possibly H3. Third, the addition of aspartate compared with the addition of buffer in pulse-chase experiments showed enhanced labeling at H2 and delabeling at H1. H1, H2, and H3 are distinct proteins (6; Fig. 1). Methyl groups were clearly being transferred. Such methyl transfer is a novel finding in the field of bacterial chemotaxis.

Both the function and mechanism of this inter-MCP methyl transfer remain obscure. It is not known whether the cheR product or a different enzyme catalyzes this transfer. If components besides the cheR product are required, they must be either membrane bound or needed in small amounts, since methylation of H2 in purified membranes occurs in vitro (6). Indeed, close analysis of Fig. 8 of reference 6, showing results for an experiment in which excess nonradioactive SAM was added after a brief period of labeling, shows that radiolabeling of H2 increases as a function of time. Such labeling must have occurred by methyl transfer. It should be possible to obtain mutants for any additional factors that may exist for this methyl transfer. If no other component is needed, then it is hard to understand why SAM cannot be the direct donor of methyl groups to H2.

It may at first seem surprising that the number of methyl groups acquired by H2 was about the same as that lost by H1, in view of the fact that methanol is produced after the addition of aspartate. Furthermore, previous results have indicated that demethylation of MCPs occurs during this time (7). More recent findings resolve these apparent discrepancies. First, it is apparent that the [3H]methionine is used up and nonradioactive methionine from internal proteolysis then becomes the source of methyl groups for MCP methylation after the addition of aspartate (5). This apparently happened in the experiments reported by Goldman et al. (7). Second, we found (Fig. 5; M. Thoelke and G. Ordal, manuscript in preparation) that the addition of attractant caused a flux of methyl groups from SAM through the MCPs to an intermediate methyl acceptor, and if these groups are nonradioactive, then apparent delabeling of MCPs will occur. Third, the amount of methanol released is small compared with the total number of methyl groups on the MCPs (D. Neftleton, Ph.D. thesis, University of Illinois, Urbana, 1986; J. Kirby, W. Bedale, M. Thoelke, and G. Ordal, unpublished data). If no net demethylation of the MCPs is occurring, the methyl groups released as methanol in response to attractant could be the result of an increased rate of methyl turnover through the MCPs.

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LITERATURE CITED