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In Vitro Methylation and Demethylation of Methyl-Accepting Chemotaxis Proteins in *Bacillus subtilis*†

Daniel J. Goldman and George W. Ordal*

ABSTRACT: *Bacillus subtilis* responds to attractants by demethylating a group of integral membrane proteins referred to as methyl-accepting chemotaxis proteins (MCPs). We have studied the methylation and demethylation of these proteins in an in vitro system, consisting of membrane vesicles, and purified methyltransferase and methylesterase. The chemoattractant aspartate was found to inhibit methylation and stimulate demethylation of MCPs. *Escherichia coli* radiolabeled membranes in the presence of *B. subtilis* enzyme do not respond to aspartate by an increase demethylation rate. We also report that *B. subtilis* MCPs are multiply methylated, demethylation resulting in slower migrating proteins on sodium dodecyl sulfate-polyacrylamide gels.

Posttranslational modification of proteins is involved in a number of regulatory processes. In bacterial chemotaxis, the level of methylation of certain integral membrane proteins, referred to as methyl-accepting chemotaxis proteins (MCPs), has been correlated with the adaptation of bacteria to various chemoeffectors (Goy et al., 1977; Goldman et al., 1982).

We have been involved in studying the response of *Bacillus subtilis* toward the amino acid attractants. We have found that all the amino acids function as attractants for *B. subtilis*. The response of the bacteria is proportional to the number of receptors occupied by the amino acid (Goldman & Ordal, 1981). In vivo methylation experiments indicate the *B. subtilis* responds to amino acid attractants by demethylating its MCPs, and it is during this increased rate of demethylation that the bacteria swim smoothly (Goldman et al., 1982). This is a unique finding in bacterial chemotaxis. The Gram-negative bacteria like *Escherichia coli* respond to amino acid attractants by an increased rate of MCP methylation (Goy et al., 1977) and a decreased rate of MCP demethylation (Toews et al., 1979).

S-Adenosylmethionine serves as the methyl donor for MCPs (Rollins & Dahlquist, 1980). A methyltransferase enzyme responsible for catalyzing this methylation has been purified from *B. subtilis* (Burgess-Cassler et al., 1982). Recently, we have purified the methylesterase responsible for catalyzing removal of these methyl groups (Goldman et al., 1984).

Utilizing an in vitro system consisting of purified enzymes and membrane vesicles, we have examined the methylation/demethylation reactions individually. We also investigated the influence of chemoattractant on these reactions. We report here that the chemoeffector aspartate stimulated MCP demethylation in vitro and also inhibited MCP methylation. Using a heterologous sytem consisting of *E. coli* membranes and *B. subtilis* methylesterase, we present evidence that the effect of aspartate on MCP methylation is mediated through the membranes. Furthermore, we present evidence that *B. subtilis* MCPs are multiply methylated.

Materials and Methods

Chemicals. *S*-Adenosyl[*methyl*-^{3}H]methionine (15 Ci/mmol) and L-[*methyl*-^{3}H]methionine (78-93 Ci/mmol) were obtained from Amersham Corp. or ICN. L-Aspartic acid potassium salt was purchased from Sigma. All other chemicals were reagent grade.

Bacterial Strains. Strain 0I 1085 is a chemotactically wild-type strain of *Bacillus subtilis*. Strain 0I 1100 was obtained from 0I 1085 by mutagenesis and is a chemotaxis mutant (Ullah & Ordal, 1981; Burgess-Cassler et al., 1982). *Escherichia coli* strain RP4612 (RP437 *cheR*) is a chemotaxis methyltransferase mutant obtained from J. S. Parkinson.

Media. Tryptone broth contains 1% tryptone and 0.5% NaCl. L broth contains 1% tryptone, 0.5% NaCl, and 0.5% yeast extract. FP buffer and MT buffer have been previously described (Burgess-Cassler et al., 1982).

Membrane Vesicle Isolation and Enzyme Purification. *B. subtilis* and *E. coli* were grown in tryptone broth overnight, then subcultured and grown in L broth to 180 Klett units (*B. subtilis*) or to A_{595} of 1-1.7 (*E. coli*) ~(2-8 × 10^8 cells/mL) at 37 °C. Cells were chilled to about 4 °C and all subsequent

† From the School of Basic Medical Sciences and the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received October 10, 1983.*

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steps performed at 0–4 °C. Cells were washed twice with 1 M KCl and once with FP buffer and resuspended in FP buffer. Cells were broken open by passage through an Aminco French pressure cell 3 times at 19,000 psi. Unbroken cells and debris were removed by centrifuging the above extract at 17000*g* for 20 min in a Beckman JA-20 rotor. Membranes were isolated by centrifugation at 120000*g* for 3–6 h in a Beckman 70 Ti rotor. The membranes were stored frozen at −70 °C in small aliquots until needed. Methyltransferase II and methylesterase were purified as described previously (Burgess-Cassler et al., 1982; Goldman et al., 1984).

In Vitro and in Vivo Methylation Assays. In vivo methylation assays were performed as described previously (Goldman et al., 1982). In vitro methylation assays consisted of 5 μL of bacterial membrane (75 μg of protein), 2 μL of 0.1 M $MgCl_2$, 10 μL of 80% glycerol, 5 μL of aspartate (final concentration 0.1 M) or buffer, and 25 μL of purified methyltransferase II (~0.25 μg of protein). Methylation was initiated by addition of 5 μL of *S*-adenosyl[*methyl*-^{3}H]-methionine (1.67 μM). Reactions were terminated by addition of 35 μL of 2× Laemmli sample buffer (Laemmli, 1970).

Samples were boiled for several minutes and applied to SDS–polyacrylamide[1] gels [10% acrylamide and 0.125% bis(acrylamide)] (Laemmli, 1970). Gels were prepared for fluorography by the method of Laskey & Mills (1975). Fluorograms were scanned with a Biomed Instruments Model SL-504-XL soft laser scanning densitometer.

In Vitro Demethylation Assays. Membranes were methylated in vitro as described above. After the desired time of methylation, 5 μL of 1 mM *S*-adenosylmethionine was added, followed by 5 μL of chemoeffector or buffer and 10 μL of methylesterase (~0.12 μg of protein). The reaction was allowed to proceed for various lengths of time and stopped by addition of 35 μL of 2× Laemmli sample buffer (Laemmli, 1970). Samples were analyzed on SDS–polyacrylamide gels as described above.

Analysis of Volatile Radioactive Products. Conway diffusion cells were employed to measure volatile radioactive product (Goldman et al., 1982). Bacteria were methylated in vivo with L-[*methyl*-^{3}H]methonine, and unreacted methionine was removed by washing the cells. Bacterial extract was prepared by breaking the cells open as described under Membrane Vesicle Isolation and Enzyme Purification, but membranes were not separated from cytoplasm.

The outer well of the Conway cell contained 45 μL of radiolabeled bacterial extract and 5 μL of 2× aspartate (0.1 M final concentration) or buffer. Opposite this bacterial extract was placed 50 μL of 2× Laemmli sample buffer and a glass bead. The center well contained 0.5 mL of water. The Conway cells were closed, Vaseline being used to obtain a tight seal. The reaction was terminated by rotating the Conway cells so the Laemmli sample buffer mixed with the bacterial extract. The cells were allowed to sit overnight and then opened, and 0.25 mL from the center well was analyzed for radioactivity by liquid scintillation spectroscopy.

Results

Effect of Attractant on MCP Methylation. All the amino acids serve as attractants for *B. subtilis* (Goldman & Ordal, 1981; Ordal & Gibson, 1977). Amino acids were found to cause a net decrease in the level of methylation of *B. subtilis* MCPs. The amino acids stimulate methanol formation, and during this increased rate of methanol formation the bacteria swim smoothly (Goldman et al., 1982).

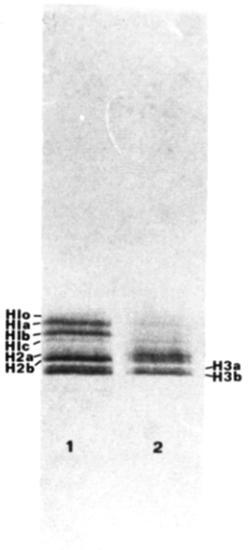

FIGURE 1: Effect of aspartate on in vivo methylation profile of *B. subtilis* MCPs. In vivo methylation experiments were performed as described under Materials and Methods. Aspartate was added to 10 times the K_d concentration (Ordal et al., 1977). (Lane 1) Buffer control; (lane 2) aspartate.

Although we know that amino acids stimulate the demethylation reaction in *B. subtilis*, it is difficult to determine the effects of amino acids on the methylation reaction in vivo. Therefore, an in vitro system was investigated that allowed us to examine the methylation or demethylation reactions individually and the effect of attractant on these reactions. We chose to study the attractant aspartate since it causes the longest adaptation time of all the amino acids and produces the greatest change in MCP methylation profiles (Goldman & Ordal, 1981; Goldman et al., 1982). Furthermore, aspartate is an effective competitor of all amino acid responses as determined by Ordal et al. (1977) with "jamming capillary assays".

An in vivo methylation profile of *B. subtilis* radiolabeled MCPs is presented for cells incubated in the absence and presence of aspartate (Figure 1). Radiolabeled MCP bands are referred to as H1o through H3b. When we originally published the results of attractants effects on MCP methylation profiles (Goldman et al., 1982), we labeled these bands as H1a through H3b. H1o and H1c were not labeled since these proteins were only lightly methylated and it was difficult to quantitate changes in their methylation level.

Purified methyltransferase II was used to methylate 0I 1100 membranes in vitro. These membranes are derived from a chemotaxis mutant (Ullah & Ordal, 1981) that lacks a functional methyltransferase II enzyme (Burgess-Cassler et al., 1982). These membranes are found to have very little if any methyltransferase II or methylesterase enzymes contaminating them (unpublished observations). Methylation reactions were stopped as a function of time and proteins resolved by SDS–PAGE. Radiolabeled proteins were visualized by fluorography, and the fluorograms were scanned with a densitometer (Figure 2). Addition of aspartate (at 90% receptor occupancy, 0.1 M) to these methylation reactions is observed to significantly decrease incorporation of [^{3}H]methyl groups into bands H1a, H1b, H2a, and H2b. Bands H1o, H3a, and H3b appear to be only slightly reduced, if at all, by the presence of aspartate. It is interesting that band H1c has a

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

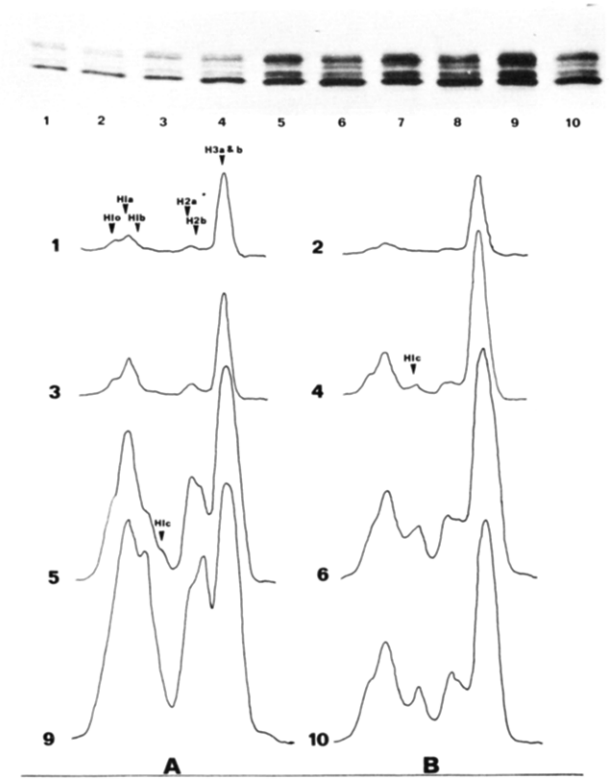

FIGURE 2: Time course of in vitro methylation; effect of aspartate on 0I 1100 membranes. In vitro methylation experiments were performed as described under Materials and Methods. Presented is the fluorogram of SDS–PAGE of radiolabeled MCPs and densitometer scans of these gels. Odd-numbered gel lanes are samples treated with buffer; even-numbered lanes are samples treated with aspartate (0.1 M): (lanes 1 and 2) 19, (lanes 3 and 4) 45, (lanes 5 and 6) 120, (lanes 7 and 8) 165, and (lanes 9 and 10) 240 min of methylation. Densitometer scans represent (A) buffer-treated samples corresponding to gel lanes 1, 3, 5, and 9 and (B) aspartate-treated samples corresponding to gel lanes 2, 4, 6, and 10.

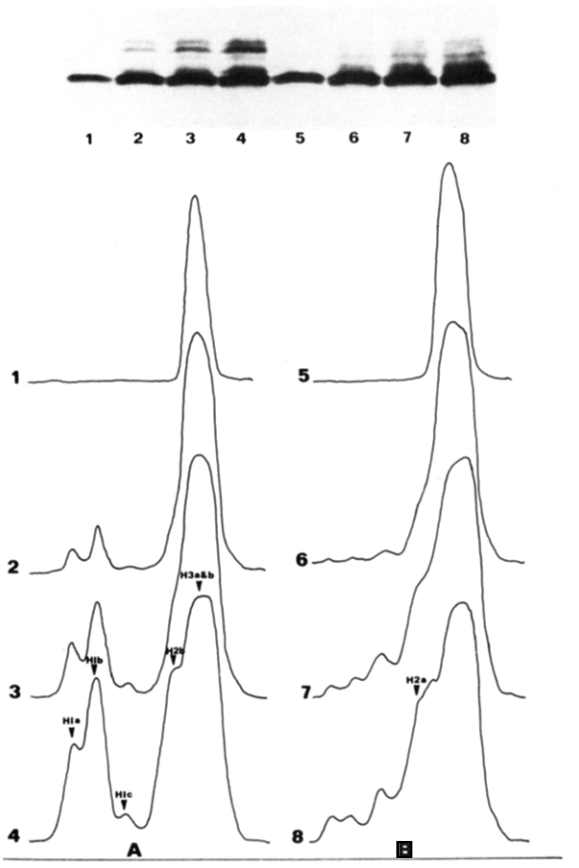

FIGURE 3: Time course of in vitro methylation; effect of aspartate on 0I 1085 membranes. In vitro methylation experiments were performed as described under Materials and Methods. Presented is the fluorogram of SDS–PAGE of radiolabeled MCPs and densitometer scans of these gels. Lanes 1–4 are buffer-treated samples, and lanes 5–8 are aspartate– (0.1 M) treated samples: (lanes 1 and 5) 10, (lanes 2 and 6) 30, (lanes 3 and 7) 60, and (lanes 4 and 8) 120 min of methylation. Densitometer scans represent (A) buffer-treated sample corresponding to lanes 1–4 and (B) aspartate-treated sample corresponding to lanes 5–8.

higher level of radiolabeled methylation when aspartate is present compared to its absence. This result implies that it is the substrates (MCPs) that are influenced by the attractant and not the enzyme.

In vivo data indicate that H2a does not significantly incorporate [^{3}H]methyl groups until one adds aspartate to the cells (Figure 1). In vitro data, 0I 1100 membranes as substrate, indicate H2a is methylated quite readily (Figure 2). If one repeats these experiments with 0I 1085 membranes, which are derived from chemotactic wild-type bacteria, radiolabeled methylation of H2a is not observed unless aspartate is added to the reaction mixture (Figure 3).

We believe H2a represents a partially methylated MCP. The 0I 1100 membranes give rise to H2a since its MCPs are totally unmethylated. The 0I 1085 membranes are already methylated with cold methyl groups. Thus, methylation reactions with radiolabeled methionine (in vivo) or *S*-adenosylmethionine (in vitro) will result in only multiply methylated MCPs, and so, one does not observe band H2a. Addition of aspartate, however, stimulates the methylesterase reaction, therefore generating partially methylated MCP and thus generating H2a. The 0I 1085 membranes are known to be contaminated with methylesterase (unpublished observations). Further evidence for H2a being a partially methylated MCP will be presented in the section discussing demethylation reactions in this paper.

Besides the effect on H2a, methylation of 0I 1085 membranes is observed to be influenced by aspartate in a similar manner as 0I 1100 membranes. Therefore, bands H1a, H1b, and H2b are reduced in their methylation level, whereas H1c has its level of methylation increased upon methylation in the presence of aspartate. These effects of aspartate on in vitro methylation generally influence the same MCP bands as influenced in vivo (Goldman et al., 1982; Figure 1).

Effect of Attractant on MCP Demethylation. In vivo experiments indicate that demethylation of *B. subtilis* MCPs can be correlated with adaptation to amino acid attractants (Goldman et al., 1982). In light of these results, we investigated demethylation in an in vitro system.

The influence of methylesterase on in vitro methylated 0I 1100 membranes was initially investigated (Figure 4). Membranes were methylated for 2 h with pure methyltransferase II. Methylesterase or buffer was then added for an additional hour. Besides stimulating demethylation of MCPs, sample treated with methylesterase also results in an increase in label in bands H1o and H2a. This can result by demethylating a multiply methylated MCP, which migrates to a new position in its partially methylated form. Alternatively methylesterase may change the specificity of the methyltransferase enzyme. We believe the former to be the true explanation of the data on the basis of the results presented so far and that presented in cold chase experiments (presented later).

Conway cells were employed to investigate the effect of aspartate on methanol evolution (Figure 5). Wild-type bacteria were methylated in vivo, washed with FP buffer, and

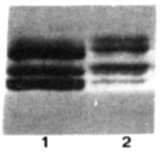

FIGURE 4: Effect of methylesterase on radiolabeled 0I 1100 membranes. Membranes were methylated for 2 h with methyltransferase II. Methylesterase (0.6 μg of protein) of buffer was then added for an additional hour. Samples were analyzed by SDS–PAGE and radiolabeled proteins visualized by fluorography: (lane 1) buffer; (lane 2) methylesterase.

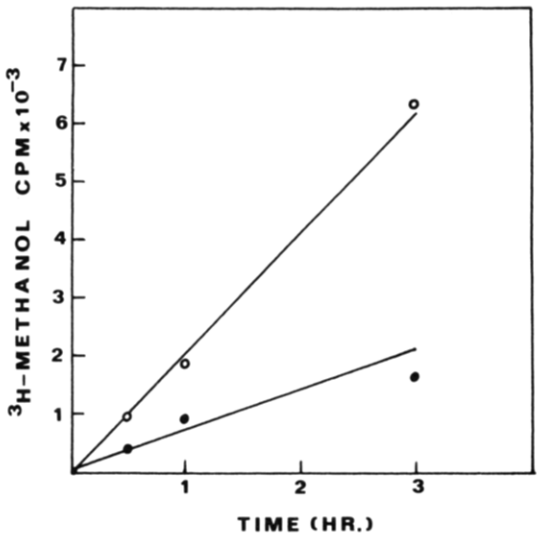

FIGURE 5: Time course of methanol evolution as a result of adding aspartate (O) or buffer (●) to bacterial extract. Aspartate was added at a final concentration of 0.1 M. Experiments used Conway diffusion cells as described under Materials and Methods.

broken open with a French pressure cell. Unlysed cells were removed by centrifugation. To the outer well of a Conway diffusion cell was added 45 μL of radiolabeled bacterial extract, and opposite this reaction mix was 50 μL of 2× Laemmli sample buffer. To the bacterial extract was added aspartate (90% receptor occupancy, 0.1 M) or buffer and the Conway cells closed. Reactions were stopped as a function of time and aliquots from the center well counted after equilibration was complete. Aspartate is observed to significantly increase the rate of methanol evolution (Figure 5). This is consistent with what is observed in vivo (Goldman et al., 1982).

In order to study the methylesterase reaction in more detail, cold chase demethylation experiments were performed and samples analyzed by SDS–PAGE (Figure 6). Radiolabeled proteins were visualized by fluorography. The methylesterase reaction without addition of effector indicates that bands H3a, H3b, and H1b lose their [^{3}H]methyl groups most readily. The gel profiles and densitometer scans of these gels indicate that as a function of time, after cold *S*-adenosylmethionine addition and methylesterase, bands H1o, H1a, and H2a increase in level of [^{3}H]methyl group (Figure 6). There is also a very faint band just below H1a that arises during the cold chase demethylation experiment. This band has not been named since it is usually difficult to resolve from H1a. Since all four of these bands arise only upon addition of methylesterase, they probably represent a paritally demethylated MCP, which migrates slower than the fully methylated form. This is consistent with *E. coli* and *Salmonella typhimurium* MCPs, where it is observed that the MCPs are multiply methylated and that the partially methylated species migrate slower in SDS–polyacrylamide gels (Boyd & Simon, 1980; Engstron & Hazelbauer, 1980).

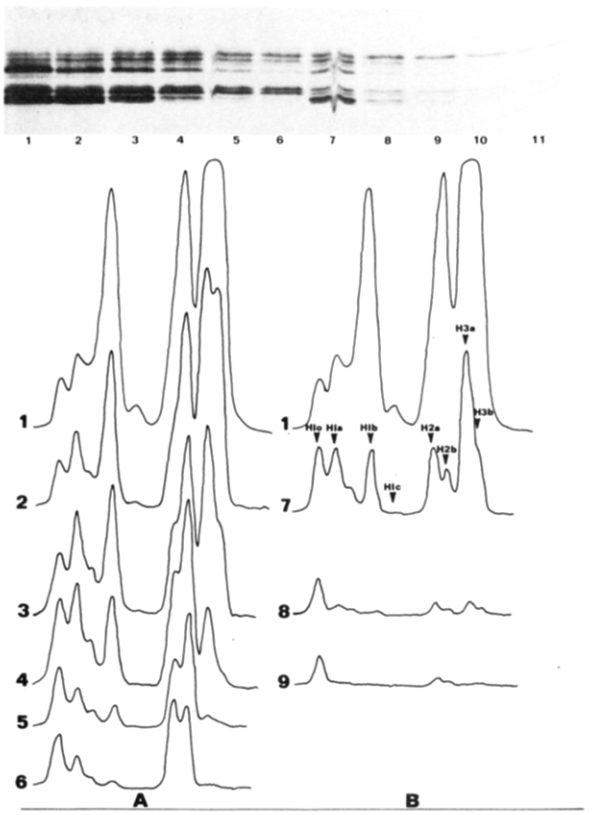

FIGURE 6: Time course of in vitro demethylation; effect of aspartate on 0I 1100 membranes. In vitro demethylation experiments were performed as described under Materials and Methods. Presented is SDS–PAGE of radiolabeled MCPs and densitometer scans of these gels. The 0I 1100 membranes were methylated for 3 h with methyltransferase II. To these radiolabeled membranes were added cold *S*-adenosylmethionine (0.1 mM), methylesterase, and buffer (lanes 2–6) or aspartate at 0.1 M final concentration (lanes 7–11). Reactions were stopped as a function of time and analyzed by SDS–PAGE: (lane 1) 3-h methylated sample; (lanes 2 and 7) 5, (lanes 3 and 8) 10, (lanes 4 and 9) 30, (lanes 5 and 10) 70, and (lanes 6 and 11) 120 min of cold chase demethylation. Densitometer scans represent (A) buffer-treated sample corresponding to lanes 1–6 and (B) aspartate-treated sample corresponding to lanes 1 and 7–9.

The effect of aspartate (90% receptor occupancy, 0.1 M) on MCP demethylation if presented in Figure 6. Aspartate is observed to stimulate the methylesterase reaction. It also influences the specificity of the methylesterase. If aspartate just resulted in an increased rate of the methylesterase reaction, one might expect the 5-min aspartate-treated sample to give an MCP profile similar to that of 30-min buffer-treated sample. What one observes is that band H3 is slightly protected relative to the other MCP bands. Therefore, bands H1a, H1b, H2a, and H2b are demethylated at a faster rate relative to H3 when aspartate is present compared to its absence.

To further investigate the generation of bands H1o, H1a, and H2a, in vitro demethylation of 01 1085 membranes was investigated. These membranes are derived from chemotactically wild-type bacteria. Membranes were methylated with pure methyltranserase II, and demethylation was monitored after addition of cold *S*-adenosylmethionine and pure methylesterase. These membranes are observed to not methylate H1o and H2a during methylation assays (Figure 3). Addition of aspartate is found to generate radiolabeled H2a (Figure 3). This may result from a demethylation of a multiply methylated MCP, since these membranes are contaminated with methylesterase enzyme. If H1o, H1a, and H2a are products of demethylation of multiply methylated MCPs, one expects that if methylesterase is added to methylated 0I 1085 membranes,

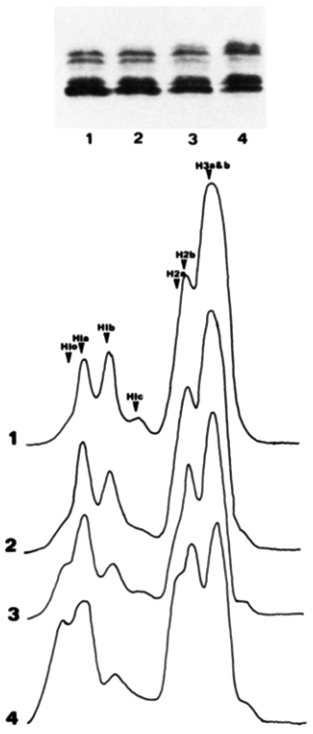

FIGURE 7: Time course of in vitro demethylation of 0I 1085 membranes. In vitro demethylation experiments were performed as described under Materials and Methods. Presented is SDS–PAGE of radiolabeled MCPs and densitometer scans of these gels. The 0I 1085 membranes were methylated for 2 h with methyltransferase II. To these radiolabeled membranes were added cold *S*-adenosylmethionine (0.1 mM), methylesterase, and buffer. Reactions were stopped as a function of time and analyzed by SDS–PAGE: (lane 1) 5, (lane 2) 10, (lane 3) 20, and (lane 4) 30 min of demethylation. Densitometer scans correspond to lanes 1–4 of the gel.

H1o, H1a, and H2a would be generated even in the absence of aspartate stimulation. This type of analysis was performed, and, as predicted, H1o, H1a, and H2a were generated by demethylation of these methylated membranes (Figure 7).

These data also imply that the methylesterase does not just remove those methyl groups most recently put on. With 0I 1085 membranes, one does not observe methylation of H1o and H2a (Figure 3), whereas with 0I 1100 membranes H1o and H2a are methylated very early during the methylation reaction (Figure 2). If one removes the radiolabeled methyl groups put on 0I 1085 membranes, partially methylated MCPs would be generated that only contain cold methyl groups and no radiolabeled proteins are generated during the demethylation reaction (Figure 7); therefore, not all the methyl groups removed are radiolabeled. This implies that at least some cold methyl groups (relatively old) are removed to generate H1o and H2a.

Cold Chase Methylation. Demethylation experiments indicate that bands H1o, H1a, and H2a represent partially methylated MCPs. One expects that if these MCP forms are methylated and cold *S*-adenosylmethionine is added and methylation allowed to continue, a transfer of label from these MCP forms to their multiply methylated counterparts would occur.

Cold chase experiments were performed on 0I 1100 membranes that were ^{3}H-methylated with methyltransferase II for 10 min (Figure 8). It is observed that during the chase with cold *S*-adenosylmethionine H1o loses radioactivity while H1a, H1b, and H2b increase their level of methylation. Thus, even though no new radiolabeled methylation can take place, we

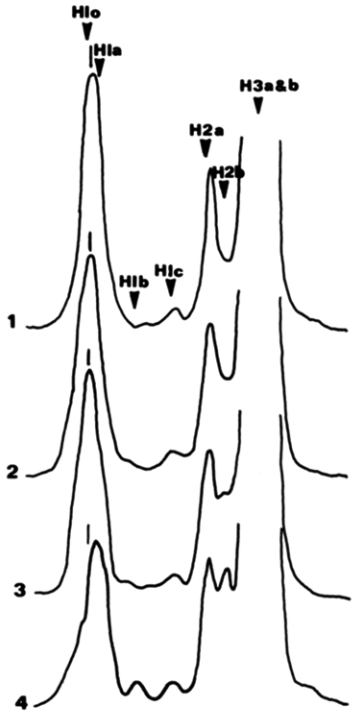

FIGURE 8: Time course of in vitro methylation, cold chase of 0I 1100 membranes. In vitro methylation was performed as described under Materials and Methods. Presented are densitometer scans of samples analyzed by SDS–PAGE. The 0I 1100 membranes were methylated for 10 min with methyltransferase II. To these radiolabeled membranes was added cold *S*-adenosylmethionine (final concentration of 0.1 mM). Reactions were stopped as a function of time after cold *S*-adenosylmethionine addition: (scan 1) 10, (scan 2) 20, (scan 3) 40, and (scan 4) 80 min after cold chase.

observe an increase in radiolabel in certain MCP forms. This is what is expected if bands H1a, H1b, and H2b represent the multiply methylated counterparts of bands H1o and H2a. Due to poor resolution of H3a and H3b, no conclusions about these MCP bands can be made.

The cold chase experiments were also performed in the presence of aspartate. Aspartate inhibits the methylation reaction (Figure 2) and inhibits the chase of label into bands H1a, H1b, and H2b (unpublished observations).

B. subtilis Methylesterase Demethylates E. coli MCPs. Purified methyltransferase II was used to methylate *E. coli* membranes derived from a methyltransferase (*cheR*) mutant (RP4612). *B. subtilis* methyltransferase II has been shown previously to be able to methylate both *B. subtilis* and *E. coli* MCPs (Burgess-Cassler & Ordal, 1983). The methylated *E. coli* membranes were incubated in cold *S*-adenosylmethionine (0.1 mM), *B. subtilis* methylesterase or buffer, and aspartate (90% receptor occupancy, 0.1 M) or buffer. Reactions were stopped as a function of time and proteins analyzed by SDS–PAGE (data not shown). *B. subtilis* methylesterase was observed to demethylate *E. coli* MCP II although the enzyme activity with the *E. coli* membranes was significantly less than when *B. subtilis* membranes were employed as substrate. Furthermore, the methylesterase reaction was not stimulated by addition of aspartate. These data imply that there is some functional homology between the *B. subtilis* and *E. coli* methylesterases and that attractants like aspartate stimulate the methylesterase reaction by influencing the MCP substrate availability.

Discussion

This paper results from a study of MCP methylation and demethylation in vitro. We find that attractants like aspartate

influence both methylation and demethylation reactions. Methylation was monitored with membranes derived from a methyltransferase mutant. These membranes lack contaminating methylesterase, probably because there is no methylated substrate available for it to recognize. Aspartate is found to significantly reduce the appearance of radiolabeled bands H1a, H1b, H2a, and H2b but enhance the appearance of H1c (Figure 2).

The demethylation reaction was also influenced by aspartate. Conway diffusion cells were used to investigate volatile radioactive product (Figure 5). Aspartate was observed to increase the rate of methanol evolution in the crude bacterial extract. Using purified components and gel analysis, we observe aspartate to stimulate demethylation of most of the membrane-bound MCPs (Figure 6). Aspartate also alters the specificity of the methylesterase reaction. In the absence of aspartate, MCP form H3 is observed to be demethylated at a faster rate than H1a, H1b, and H2b. If aspartate is present, one observes an increased rate of demethylation of bands H1a, H1b, and H2b relative to H3 (Figure 6).

We have shown previously that amino acid attractants like aspartate stimulate the demethylation reaction (Goldman et al., 1982). The MCP profile of aspartate-treated cells in vivo is presented in Figure 1. One observes that bands H1a, H1b, and H2b have decreased methylation levels upon aspartate stimulation. These are the same bands found to be most sensitive to aspartate in vitro (Figures 2, 3, and 6). Thus, it is likely that the in vivo effects of aspartate are a result of both changes in methylation and demethylation reactions.

These in vitro results are consistent with those found in vivo for effect of amino acids on MCP methylation in *B. subtilis* (Goldman et al., 1982). These results further confirm the finding that *B. subtilis* responds to attractants by stimulating the methylesterase reaction. *E. coli* responds to amino acid attractants by an increase in protein methylation (Kort et al., 1975; Springer et al., 1977; Silverman & Simon, 1977; Goy et al., 1977; Kondoh et al., 1979). It also was observed that in *E. coli* attractant stimulation caused an inhibition of the methylesterase reaction in vivo and during the length of time the demethylation reaction was inhibited the bacteria swam smoothly (Toews et al., 1979). Studies in an in vitro system with this organism indicate that attractant causes an inhibition of the methylesterase reaction and stimulates the methyltransferase reaction (Kleene et al., 1979). Thus, both the in vivo and in vitro effects of amino acid attractants in *B. subtilis* are the opposite of those found in *E. coli*.

The in vitro experiments presented in this paper also indicate that *B. subtilis* MCPs are multiply methylated. Evidence for this conclusion comes mainly from demethylation experiments. It is observed that methylesterase addition to prelabeled 0I 1100 or 1085 membranes results in generation of MCP bands H1o, H1a, and H2a (Figures 4, 6, and 7). Aspartate stimulates the methylesterase reaction and is observed to stimulate generation of bands H1o, H1a, and H2a (Figure 6). These reactions are carried out in approximately a 100-fold excess of unlabeled *S*-adenosylmethionine. Control experiments indicate that no new methylation takes place under these conditions and that generation of label in bands H1o, H1a, and H2a is dependent upon methylesterase being present. We report that during methylation of wild-type membranes addition of aspartate resulted in generation of H2a (Figure 3). We believe that this is due to an aspartate-stimulated methylesterase contaminating these membranes. Thus, *B. subtilis* MCPs are multiply methylated, and demethylation of these proteins results in a slower mobility on SDS–polyacrylamide gels. This is consistent with the results found in *E. coli* and *S. typhimurium*, where MCPs are found to be multiply methylated and have a decreased mobility, upon demethylation, in SDS–polyacrylamide gels (Boyd & Simon, 1980; Engstrom & Hazelbauer, 1980; Kehrey et al., 1983). Further evidence for *B. subtilis* MCPs being multiply methylated comes from cold chase methylation experiments (Figure 8). We find that one can chase the label out of bands H1o and H2a into H1a, H1b, and H2b. If the experiments are repeated with aspartate added during the cold chase, generation of MCPs H1a, H1b, and H2b is not observed (unpublished observations). This is consistent with the finding that aspartate inhibits the methyltransferase reaction (Figures 2 and 3).

These data imply that the number of *B. subtilis* MCPs is less than the number of radiolabeled proteins observed on a SDS–polyacrylamide gel. In order to determine the number of gene products that are MCPs and therefore determine which bands on a gel represent the same protein, MCP mutants are required. Since we do not have these mutants for analysis, one can only infer from the experiments presented about which MCPs represent the same gene product. On the basis of demethylation time-course experiments, it appears that bands H1o, H1a and H1b represent one gene product and bands H2a and H2b represent the second gene product (Figures 6 and 7).

Does aspartate influence the methylesterase reaction by interaction with its receptor, which results in MCPs becoming better substrates for the methylesterase? Why does aspartate result in a stimulation of the methylesterase reaction in *B. subtilis* but inhibit this reaction in *E. coli*? These questions were investigated with methylated membranes derived from an *E. coli* methyltransferase mutant. The effect of *B. subtilis* methylesterase on MCP demethylation in the presence and absence of aspartate was determined. We find that *B. subtilis* methylesterase does recognize *E. coli* methylated membranes as substrate. This result is not surprising in light of the fact that the methyltransferases from the two organisms are functionally homologous (Burgess-Cassler & Ordal, 1982). Addition of aspartate, however, did not stimulate the methylesterase reaction. This result is consistent with the idea that the *E. coli* membranes respond to aspartate by causing MCPs to assume a conformation that makes them a poor substrate for methylesterase while aspartate causes *B. subtilis* MCPs to become better substrates for the enzyme.

The in vitro assays reported here have allowed us to study the methylation and demethylation reactions individually, under well-defined conditions. We utilized this system to study the effects of chemoeffectors on these reactions. We believe this in vitro system will provide a convenient assay for other cellular components that may influence these methylation/demethylation reactions.

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Registry No. MCP-*O*-methyltransferase II, 83137-81-9; aspartic acid, 56-84-8; *O*-demethylase, 9012-67-3.

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Dynamic Equilibrium between the Two Conformational States of Spin-Labeled Tropomyosin†

Philip Graceffa* and Sherwin S. Lehrer

ABSTRACT: Tropomyosin was labeled with a maleimide nitroxide spin-label attached to cysteine-190 via a succinimido ring which was subsequently opened by incubation at alkaline pH. Electron spin resonance (ESR) spectra showed a temperature-dependent equilibrium, below the main unfolding transition of tropomyosin, between labels which were restricted in their motion (strongly immobilized), predominating at low temperatures, and those which were highly mobile (weakly immobilized), predominating at higher temperatures. These label states were associated with two protein states from a comparison of the ESR spectral changes with the thermal unfolding profile of tropomyosin. The strongly immobilized labels were associated with the completely folded molecule and the weakly immobilized labels with a partially unfolded (in the cysteine-190 region) state which is an intermediate in the thermal unfolding of tropomyosin. A spectral subtraction technique was used to measure the concentration ratio of strongly and weakly immobilized labels from which an equilibrium constant, K, was determined at different temperatures. A linear van't Hoff plot was obtained, indicating that the spin-labeled protein is in thermal equilibrium between these two conformational states with $\Delta H = 17$ kcal/mol, $\Delta S = 56$ cal/(deg·mol), and $K = 1.0$ at 34 °C. An upper limit of 10^7 s^{-1} for the conformational fluctuation was estimated from the shapes and separation of the two ESR spectral components. In contrast to the label with the opened succinimido ring, the spin-label with an intact succinimido ring remained strongly immobilized on the protein, indicating that in the partially unfolded state the molecule retains structure in the cysteine-190 region.

Tropomyosin, a regulatory muscle protein, is composed of two parallel, coiled-coil α-helical polypeptide chains in a rod-shaped structure with a high α-helical content (Caspar et al., 1969). Rabbit skeletal tropomyosin contains two types of chains, α and β, with an α/β ratio of roughly 4/1 (Cummins & Perry, 1973) which are combined to form α-α and α-β tropomyosin molecules (Eisenberg & Kielley, 1974; Yamaguchi et al., 1974; Lehrer, 1975). Both chains have a very similar amino acid sequence of 284 residues, with the α chain having one cysteine residue at position 190 in the C-terminal half of the molecule and the β chain containing cysteine residues at positions 190 and 36 (Mak et al., 1980). The two chains of tropomyosin are in register since they can form an interchain disulfide bond at cysteine-190 (Johnson & Smillie, 1975; Lehrer, 1975; Stewart, 1975).

Although tropomyosin has generally been considered to be a rigid rod, evidence has accumulated which shows that tropomyosin has regions of lower stability which are thought to impart some degree of flexibility to the molecule. The molecule unfolds, with increasing temperature or concentration of denaturant, in two distinct stages (Woods, 1969; Pont & Woods, 1971; Satoh & Mihashi, 1972). The first stage, the pretransition, appears to involve unfolding of the C-terminal half of the molecule, and the second stage, the main transition, involves the complete unfolding of the molecule (Chao & Holtzer, 1975; Woods, 1977; Betcher-Lange & Lehrer, 1978; Lehrer, 1978; Pato & Smillie, 1978; Potekhin & Privalov, 1978). Woods (1976) has described this as an equilibrium between the native molecule, N, a partially unfolded (in the C-terminal half) intermediate state, X, and the completely denatured tropomyosin, D; i.e., $N \rightleftharpoons X \rightleftharpoons D$. Studies of the fluorescence of probes attached to cysteine-190 (Graceffa &

† From the Department of Muscle Research, Boston Biomedical Research Institute, Boston, Massachusetts 02114. *Received September 15, 1983.* This work was supported by Grants HL-22461 (S.S.L.) and AM-30917 (P.G.) from the National Institutes of Health. A preliminary report of this research was presented at the 26th Annual Biophysical Society Meeting (Graceffa & Lehrer, 1982).

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