Sharp, T. R., & Benkovic, S. (1979) *Biochemistry 18*, 2910–2916.
Shimizu, T. (1979a) *J. Biochem. 85*, 1421–1426.
Shimizu, T. (1979b) *J. Biochem. 86*, 1139–1145.
Shimizu, T., & Kimura, I. (1974) *J. Biochem. 76*, 1001–1008.
Shimizu, T., & Kimura, I. (1977) *J. Biochem. 82*, 165–173.
Shimizu, T., & Johnson, K. A. (1983a) *J. Biol. Chem. 258*, 13833–13840.
Shimizu, T., & Johnson, K. A. (1983b) *J. Biol. Chem. 258*, 13841–13848.
Shimizu, T., & Katsura, T. (1988) *J. Biochem. 103*, 99–105.
Shimizu, T., & Johnson, K. A. (1989) *Biochemistry* (in press).
Shimizu, T., Kaji, K., & Kimura, I. (1977) *J. Biochem. 82*, 1145–1153.
Sleep, J. A., Hackney, D. D., & Boyer, P. D. (1978) *J. Biol. Chem. 253*, 5235–5238.
Sleep, J. A., Hackney, D. D., & Boyer, P. D. (1980) *J. Biol. Chem. 255*, 4094–4099.
Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54–56.
Weeds, A. G., & Pope, B. (1976) *J. Mol. Biol. 111*, 129–157.

Novel Methyl Transfer during Chemotaxis in *Bacillus subtilis*†

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ABSTRACT: If *Bacillus subtilis* is incubated in radioactive methionine in the absence of protein synthesis, the methyl-accepting chemotaxis proteins (MCPs) become radioactively methylated. If the bacteria are further incubated in excess nonradioactive methionine ("cold-chased") and then given the attractant aspartate, the MCPs lose about half of their radioactivity due to turnover, in which lower specific activity methyl groups from *S*-adenosylmethionine (AdoMet) replace higher specific activity ones. Due to the cold-chase, the specific activity of the AdoMet pool is reduced at least 2-fold. If, later, the attractant is removed, higher specific activity methyl groups return to the MCPs. Thus, there must exist an unidentified methyl carrier that can "reversibly" receive methyl groups from the MCPs. In a similar experiment, labeled cells were transferred to a flow cell and exposed to addition and removal of attractant and of repellent. All four kinds of stimuli were found to cause methanol production. Bacteria with maximally labeled MCPs were exposed to many cycles of addition and removal of attractant; the maximum amount of radioactive methanol was evolved on the third, not the first, cycle. This result suggests that there is a precursor–product relationship between methyl groups on the MCPs and on the unidentified carrier, which might be the direct source of methanol. However, since no methanol was produced when a methyltransferase mutant, whose MCPs were unmethylated, was exposed to addition and removal of attractant or repellent, the methanol must ultimately derive from methylated MCPs.

In a number of species of bacteria, certain membrane proteins, termed methyl-accepting chemotaxis proteins (MCPs), act as transducers which undergo reversible methyl esterification of glutamate residues in response to binding of chemical attractants or repellents (Goldman et al., 1982; Kort et al., 1975; Springer et al., 1977; Kleene et al., 1977; Van Der Werf & Koshland, 1977). In *Escherichia coli*, this modification is thought to play a role in adaptation (Springer et al., 1977; Goy et al., 1977). During adaptation, the bacteria swim smoothly, methylation of MCPs occurs, and methanol production ceases. Eventually, the bacteria return to random behavior, net methylation of MCPs no longer changes, and the basal level of methanol production resumes. On removal of attractant, the bacteria transiently tumble, MCPs are demethylated, and methanol production is briefly enhanced. Repellents have the opposite effects as attractant on methylation of MCPs and on methanol production (Springer et al., 1977; Toews et al., 1979; Kehry et al., 1984). The methyl donor for the MCPs is *S*-adenosylmethionine (AdoMet) (Springer & Koshland, 1977).

In vitro experiments in *Bacillus subtilis* have shown that the methyl donor for MCPs is AdoMet (Burgess-Cassler et al., 1982) and that glutamate side chains are methylated (Ahlgren & Ordal, 1983). However, the metabolic events that occur following addition of attractant are quite different in the two species of bacteria. Addition of the attractant aspartate to *B. subtilis* causes an immediate redistribution of methyl groups, a flux of methyl groups through the MCPs, and a period of increased methanol production (Goldman et al., 1982; Thoelke et al., 1988). The fact that radioactive methyl groups are immediately lost from MCPs but only gradually emerge as methanol implies that methyl groups are first transferred to an intermediate carrier before evolving as methanol (Thoelke et al., 1988).

Thus, the effect of addition of attractant to *B. subtilis* is different from its effect on *E. coli*. We next wished to investigate the effects of removal of attractant on MCP methylation and methanol formation.

Experimental Procedures

Strains. B. subtilis strain OI1085 (*trpF7 hisH2 metC*) is wild type for chemotaxis (Ullah & Ordal, 1981). Strain OI1100 is its *cheR* derivative and lacks methyltransferase (Ullah & Ordal, 1981).

E. coli strain RP437 (F^- *thi thr leu his met eda-50 thr-1 rpsL*) is wild type for chemotaxis (Slocum & Parkinson, 1985).

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

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

In Vivo Methylations. In vivo methylation was performed as described by Ullah and Ordal (1981), with the following changes: cells were grown to 180 Klett units (red filter), washed, and resuspended in CB at an A_{600} of 1.0. The suspension was incubated at 25 °C with shaking. Methylation was initiated by addition of [^{3}H]methionine (10 μCi/mL). After 8 min, excess nonradioactive methionine was added (10 μM). Two minutes later, attractant was added. At various times before and after attractant addition, samples were removed from the reaction mixture and frozen in a dry ice–acetone bath to halt all cellular reactions. To remove attractant, the mixture was then filtered on a 0.45-μm Millipore filter and washed with CB containing nonradioactive methionine (10 μM). The filtered cells were then resuspended in their original volume in CB with excess nonradioactive methionine. Samples were once again removed at various times and frozen. Later, they were thawed at 4 °C, and lysozyme was added to a final concentration of 1 mg/mL. After 30 min of incubation at 4 °C, cells were pelleted.

In order to achieve steady-state labeling of the MCPs, cells were grown and washed as before and given 10 μCi/mL [^{3}H]methionone and nonradioactive methionine (10 μM final concentration). Cells were incubated at 37 °C for 25 min.

E. coli was grown in LBr to 90 Klett units at 30 °C and then washed in the same manner as for *B. subtilis*. Cells were labeled at 30 °C for 15 min with 20 μCi/mL [^{3}H]methionine.

SDS–Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed by the method of Laemmli (1970). After the addition of 100 μL of Laemmli sample buffer to the pelleted cells, the samples were boiled for 7 min and fractionated on an SDS gel made from 10% acrylamide and 0.125% bis(acrylamide). Gels were prepared for fluorography by the method of Laskey and Mills (1975).

Continuous-Flow Assay for Methanol Evolution. A system based on one by Kehry et al. (1984) has been described previously (Thoelke et al., 1987). Cells were labeled as described above for in vivo methylations and then placed in a 0.45-μm Millipore filter unit. Buffer was then pumped past the cells at a fixed rate and collected in a fraction collector. Samples were assayed for volatile labeled product and conductivity. The buffer reservoirs were switched so that the cells experienced addition and removal of attractant or repellent.

A similar assay was performed on *E. coli* using the same procedure.

Assay of AdoMet. Bacteria were treated as for in vivo methylation except that the pellets were extracted with butanol–acetic acid–water (120:30:50) and applied to Whatman P-81 paper. The chromatogram was developed overnight using 0.5 M sodium acetate, pH 5.5. The R_f of AdoMet was 0.4, and that of methionine was 0.85.

Results

Effect of Addition and Removal of Attractant on MCPs. Addition of the attract aspartate causes a rapid redistribution of methyl groups on the MCPs with a loss of groups from the MCP H1 and an increase at the MCP H2 (Goldman et al., 1982; Thoelke et al., 1988; Bedale et al., 1985). In "cold-chase" conditions, this redistribution is accompanied by an overall loss of about 50% of the label in the MCP region (Thoelke et al., 1988) (Figure 1). This overall loss of label is not seen in "non-chase" experiments and is due to increased turnover of methyl groups on the MCPs (Thoelke et al., 1988; Bedale et al., 1988).

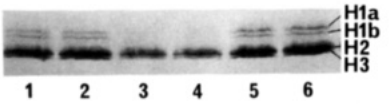

FIGURE 1: Fluorogram of the effect of aspartate addition and removal on MCP methylation. Experiments were performed as described under Experimental Procedures. Cells were incubated in radioactive methionine. At 9 min, excess (10 μM) nonradioactive methionine was added. At 11 min, 30 s, 0.1 M aspartate was added and at 12 min, 30 s was removed. Samples were taken at the following times: lanes 1 and 2, 11 min; lanes 3 and 4, 12 min; lanes 5 and 6, 14 min. Only the MCP region of the fluorogram is pictured.

The fact that radioactive methyl groups were lost from MCPs only when excess nonradioactive methionine was added before the attractant implies that the methyl donor, AdoMet, had become lower in specific activity. We verified this decrease in specific activity by chromatographing cell extracts incubated with [^{3}H]methionine (see Experimental Procedures). The addition of 0.1 μM [^{3}H]methionine caused radiolabeling of AdoMet, which was detected on a chromatogram. Subsequent addition of 10 μM [^{3}H]methionine of the same specific activity as the original caused the number of counts in AdoMet to double in 2 min and change little during the next several minutes, suggesting that the size of the AdoMet pool had increased by about 2-fold. If, however, 10 μM nonradioactive methionine was added instead, the number of counts of AdoMet was unchanged. Since the size of the AdoMet pool had approximately doubled, the specific activity of AdoMet had therefore decreased by a corresponding amount.

When aspartate was removed from cells that had been first labeled and then chased, the specific activity of methyl groups on the MCPs was restored to the prestimulus levels despite the fact that excess nonradioactive methionine was still present. In other words, the presence of excess nonradioactive methionine, which had, by reduction of the specific activity of AdoMet, allowed aspartate to cause "delabeling" of the MCPs, especially noticeable in H1a and H1b, did not prevent "relabeling". The simplest interpretation is that the radioactive methyl groups must have arrived from another source, one whose specific activity was equally as high as that of the MCPs just before addition of aspartate.

Effect of Positive and Negative Stimuli on Methanol Evolution. We also studied the effect of removal of attractant on methanol formation in *B. subtilis*. In *E. coli*, the removal of attractant has the opposite effect on addition of attractant; addition causes a transient drop in the rate of methanol formation, and removal causes a transient increase in the rate (Toews et al., 1979; Kehry et al., 1984).

In *B. subtilis*, the removal of attractant did not cause the opposite of addition of attractant. Using a continuous-flow assay, we see that the removal of aspartate also caused a transient increase in methanol production (Figure 2). This peak of methanol arose as the aspartate level was still decreasing (measured by conductivity; data not shown) and therefore cannot be due to any "overshoot" effect (Berg & Tedesco, 1974; Block et al., 1983). The addition and removal of the repellent chlorpromazine also caused methanol formation (Figure 2) (Thoelke et al., 1987). Therefore, whereas in *E. coli* positive stimuli inhibit methanol production and neg-

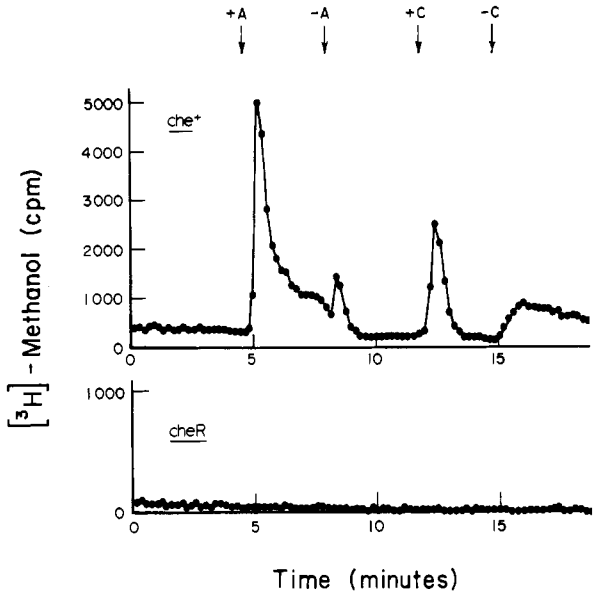

FIGURE 2: Flow assay showing the effects of attractant and repellent addition and removal in wild type and methyltransferase mutant. Experiment performed as described under Experimental Procedures. Labeled cells (50 μCi/mL [^{3}H]methionine) were loaded onto a filter and had buffer flushed past to remove any unincorporated [^{3}H]-methionine. Samples were collected every 12 s. They were assayed for labeled volatile product. At the times indicated by arrows, the buffer reservoir was switched to one containing 23 mM aspartate (+A), back to buffer (−A), to 83 μM chlorpromazine (+C), and finally back to buffer (−C). 23 mM aspartate is 70% receptor occupancy, and 83 μM chlorpromazine is the minimum concentration that prevents smooth swimming caused by 23 mM aspartate (Thoelke et al., 1987). Upper panel, OI1085 (*che*$^+$); lower panel, OI1100 (*cheR*).

ative stimuli enhance it, in *B. subtilis* all stimuli, both positive and negative, cause an increase in methanol production.

Methanol Evolution in cheR Mutant. In order to investigate the source of methyl groups for methanol production, a similar flow assay was performed on OI1100. This strain lacks the chemotactic methyltransferase and has unmethylated MCPs (Ullah & Ordal, 1981). Cells were labeled with [^{3}H]-methionine and subjected to addition and removal of attractant and repellent. However, no stimulus produced methanol. We conclude that all methanol produced by a chemotactic stimulus in wild type must ultimately derive from methyl groups on MCPs.

Effect of Multiple Attractant Exposures on Methanol Evolution. We wished to test the effect of multiple exposures of aspartate on methanol production in a flow assay. In order to ensure that the specific activities of the AdoMet pool and MCPs had stabilized and were identical, cells were given 10 μM [^{3}H]methionine for 25 min. The cells were transferred to a flow apparatus and, in the presence of nonradioactive methionine, were subjected to repeated cycles of addition and removal of aspartate. During the first three cycles, the amount of radioactive methanol monotonically increased for both addition and removal and thereafter declined (Figure 3). As in Figure 1, this finding is incompatible with exclusively "forward" transfer of methyl groups from AdoMet to MCPs to methanol since once the cells had been transferred to nonradioactive methionine the specific activity of the AdoMet pool would start to decline. These results are compatible with the transfer of methyl groups from the MCPs to one or more acceptors, which might serve as the direct source(s) of methanol.

Effects of Repeated Repellent Exposures on E. coli. A similar flow assay was performed on *E. coli* to compare to the above experiment. It has been shown that several labeled

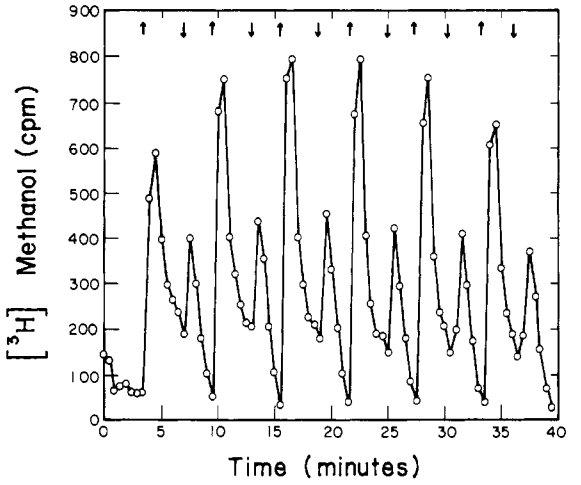

FIGURE 3: Flow assay showing the effect of multiple exposures of attractant on methanol production. Experiment performed as described in the legend to Figure 2. Cells were labeled to steady state as described under Experimental Procedures. Buffer reservoirs were changed to expose the cells to six rounds of addition and removal of aspartate (0.1 M, 90% receptor occupancy) (Goldman & Ordal, 1981). (↑) Addition of aspartate; (↓) removal of aspartate.

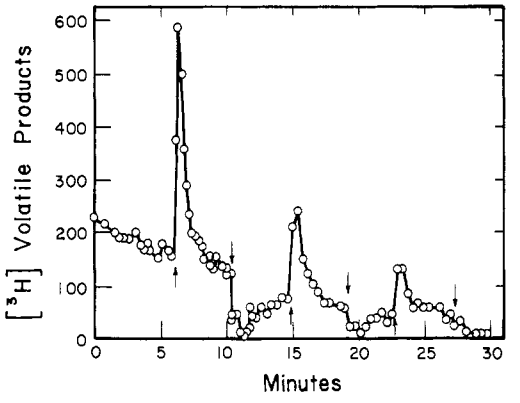

FIGURE 4: Flow assay showing the effect of multiple repellent exposures on volatile labeled product in *E. coli*. Experiment performed as described under Experimental Procedures. Repellent (0.01 M leucine) was added and removed from cells 3 times. (↑) Addition of leucine; (↓) removal of leucine.

volatile products are generated when *E. coli* is given [^{3}H]-methionine but that methanol is the only one whose generation is affected by chemoattractants (Toews et al., 1979). *E. coli* showed a rapid decay in base line before the repellent was added, indicating a more rapid basal turnover of methyl groups in the system (Figure 4). As expected, addition of repellent (0.1 M leucine) caused a period of increased methanol evolution, and removal caused a decreased evolution. The amount of labeled volatile products released upon multiple exposures to the repellent decayed rapidly. This supports the contention that the number of methyl groups released is a significant fraction of the total and that these may be released from MCPs directly to form methanol. These results are consistent with the source of remethylation of MCPs being a low specific activity methyl donor, presumably low specific activity AdoMet.

Effect of Repellent Addition on MCPs. Both addition and removal of the repellent chlorpromazine caused methanol production in *B. subtilis* (Figure 2). Other repellents have the same effect (M. Thoelke and G. Ordal, unpublished results). Many repellents appear to interact within the membrane at specific receptors that differ from the MCPs (Ordal & Villani, 1980; Ordal, 1976). It has been shown previously

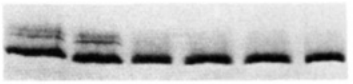

FIGURE 5: Effect of repellent addition on attractant-treated MCP methylation. Experiment performed as described under Experimental Procedures. Cells were incubated in radioactive methionine. At 6 min, excess (10 μM) nonradioactive methionine was added. At 8 min, 20 s, 0.1 M aspartate was added. At 9 min, 83 μM chlorpromazine was added. Samples were taken at the following times: lanes 1 and 2, 8 min; lanes 3 and 4, 8 min, 30 s; lanes 5 and 6, 10 min.

that addition of repellents has no significant effect on the extent or distribution of methylation of MCPs in "nonchase" conditions (Thoelke et al., 1987). Since the removal of attractant causes a relabeling of the MCPs, we attempted to determine whether a negative response due to addition of repellent subsequent to attractant addition would have this effect in "chase" conditions.

After addition of the attractant aspartate at 90% receptor occupancy, the MCPs underwent a loss of labeling as seen before (Figures 1 and 5, lanes 3 and 4 of each). Upon subsequent addition of the repellent chlorpromazine [83 μM; see legend to Figure 2 and Thoelke et al. (1987)], there is no change in either the profile or the extent of methylation of MCPs (Figure 5, lanes 5 and 6). In chase conditions, repellent added to cells in the absence of attractant does not cause any turnover-induced loss of label from the MCPs (data not shown). The negative stimulus that the addition of repellent imparts does not evoke the identical metabolic process as the negative stimulus of attractant removal. Indeed, the repellents do not seem to affect the extent or distribution of methyl groups on MCPs (Thoelke et al., 1987).

Discussion

The most striking finding of this paper is that, in a cold-chase experiment, addition of attractant caused loss of half the radioactive methyl groups from the MCPs and removal of the attractant caused their return. Under the conditions of the experiment, the specific activity of the AdoMet, known from in vitro experiments to be a methyl donor for MCPs (Burgess-Cassler et al., 1982), was shown to be at least 2-fold lower. The fact that the presence of excess nonradioactive methionine caused an approximately 2-fold reduction of specific activity of methyl groups on the MCPs compared with its absence is also consistent with a reduced specific activity of the AdoMet pool caused by the excess nonradioactive methionine. We deduce that methyl (as methyl or methoxy) groups "reversibly" are able to flow from MCPs to an unidentified carrier. Such a carrier has already been postulated to account for the immediate loss of radioactive methyl groups for MCPs but gradual evolution of radioactive methanol after addition of attractant (Thoelke et al., 1988).

The second intriguing finding was that methanol production increased on removal of aspartate as well as on addition to it. Thus, all stimuli or addition or removal of attractant or repellent (Thoelke et al., 1987) (Figure 2) causes methanol formation. In *E. coli*, only negative stimuli, addition of repellent, or removal of attractant causes methanol formation; positive stimuli actually block even basal methanol formation (Toews et al., 1977; Kehry et al., 1984).

In *B. subtilis*, even though both attractant and repellent cause methanol formation when added individually, they do not when added together at the "cross-over concentrations" (Thoelke et al., 1987). These are the concentrations at which simultaneous addition does not affect behavior. It thus appears that increased methanol formation is associated with stimulus-induced nonrandom motility and reflects demethylation reactions that bring about adaptation.

The question of the source of methyl groups for methanol may also be raised. Since stimulus-induced methanol formation does not occur in strain OI1100, all methyl groups must ultimately derive from the MCPs. The experiment shown in Figure 3 provides evidence as to the likely origin of the methanol. Cells were labeled to steady-state specific activity of MCPs, transferred to a flow apparatus, and flushed with nonradioactive methionine. Thus, any additions and removals of aspartate should have caused progressive reductions of the specific activity of MCP-associated methyl groups. In reality, the specific activity of methanol increased through several rounds of aspartate stimulus before ultimately declining. This result might be expected if the proximate source of the methyl groups was an unidentified methyl carrier whose initial specific activity of methyl groups was lower than that of the MCPs at the time of the initial addition of aspartate. This would be true if there were a precursor–product relationship between the methyl groups on the MCPs and those on this carrier. Then, the specific activity of the methanol would simply reflect the specific activity of methyl groups on this carrier, their presumed source.

It should be noted that the time course of methanol evolution from *E. coli* was very different (Figure 4). In this case, the amount of methanol released for successive stimuli (addition and removal of the repellent leucine) was much reduced each time, and the background rate of methanol released also dropped rapidly. Thus, there is no evidence for such a methyl carrier in *E. coli*, and any remethylation of MCPs seems to utilize low specific activity AdoMet as the methyl donor.

The complexity of the system is also demonstrated by the effects of repellent addition after attractant addition in "chase" conditions. This negative signal did not act in the same manner as removal of attractant. No return of label to the MCPs was seen nor were radioactive methyl groups lost upon repellent addition: that is, there is no flux of (low specific activity) methyl groups through the MCPs. Thus, repellent may act to demethylate a methyl carrier to yield methanol without any direct effect on the MCPs.

Finally, the repellent experiments raise the issue of the number of methyl carriers. We cannot say that the carrier involved for addition of repellent is the same one involved for removal of attractant nor, since removal of attractant causes both remethylation of MCPs and generation of methanol, can be say that only a single carrier is involved. Future biochemical investigation is needed to resolve these issues.

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Registry No. Methanol, 67-56-1.

References

Ahlgren, J. A., & Ordal, G. W. (1983) *Biochem. J. 213*, 759–763.

Bedale, W. A., Nettleton, D. O., Sopata, C. S., Thoelke, M. S., & Ordal, G. W. (1988) *J. Bacteriol. 170*, 223–227.

Berg, H. C., & Tedesco, P. M. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1388–1392.

Block, S. M., Segall, J. E., & Berg, H. C. (1983) *J. Bacteriol. 154*, 312–323.

Burgess-Cassler, A., Ullah, J. A. H., & Ordal, G. W. (1982) *J. Biol. Chem. 257*, 8412–8417.

Goldman, D. J., & Ordal, G. W. (1981) *J. Bacteriol. 147*, 267–270.

Goldman, D. J., Worobec, S. W., Seigel, R. B., Hecker, R. V., & Ordal, G. W. (1982) *Biochemistry 21*, 915–920.
Goy, M. F., Springer, M. S., & Alder, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4964–4968.
Kehry, M. R., Doak, T. C., & Dahlquist, F. W. (1984) *J. Biol. Chem. 259*, 11828–11835.
Kleene, S. J., Toews, M. L., & Adler, J. (1977) *J. Biol. Chem. 252*, 3214–3218.
Kort, E. N., Goy, M. F., Larsen, S. H., & Adler, J. (1975) *Proc. Natl. Acad. Sci. U.S.A 72*, 3939–3943.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335–341.
Ordal, G. W. (1976) *J. Bacteriol. 126*, 72–79.
Ordal, G. W., & Goldman, D. J. (1975) *Science 189*, 802–805.
Ordal, G. W., & Villani, D. P. (1980) *J. Gen. Microbiol. 118*, 471–478.
Slocum, M. S., & Parkinson, J. S. (1985) *J. Bacteriol. 163*, 586–594.
Springer, M. S., Goy, M. F., & Adler, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 183–187.
Springer, W. R., & Koshland, D. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 533–537.
Thoelke, M. S., Bedale, W. A., Nettleton, D. O., & Ordal, G. W. (1987) *J. Biol. Chem. 262*, 2811–2816.
Thoelke, M. S., Parker, H. M., Ordal, E. A., & Ordal, G. W. (1988) *Biochemistry 27*, 8453–8457.
Towes, M. L., Goy, M. F., Springer, M. S., & Adler, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5544–5548.
Ullah, A. J. H., & Ordal, G. W. (1981) *J. Bacteriol. 145*, 958–965.
Van Der Werf, P., & Koshland, D. E. (1977) *J. Biol. Chem. 252*, 2793–2795.

Roles of Colchicine Rings B and C in the Binding Process to Tubulin†

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ABSTRACT: The interactions of tubulin with colchicine analogues in which the tropolone methyl ether ring had been transformed into a *p*-carbomethoxybenzene have been characterized. The analogues were allocolchicine (ALLO) and 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl (TCB), the first being transformed colchicine and the second transformed colchicine with ring B eliminated. The binding of both analogues has been shown to be specific for the colchicine binding site on tubulin by competition with colchicine and podophyllotoxin. Both analogues bind reversibly to tubulin with the generation of ligand fluorescence. The binding of ALLO is slow, the fluorescence reaching a steady state in the same time span as colchicine; that of TCB is rapid. The displacement of ALLO by podophyllotoxin proceeds with a half-life of ca. 40 min. Binding isotherms generated from gel filtration and fluorescence measurements have shown that both analogues bind to tubulin with a stoichiometry of 1 mol of analogue/mol of α–β tubulin. The equilibrium binding constants at 25 °C have been found to be $(9.2 \pm 2.5) \times 10^5$ M^{-1} for ALLO and $(1.0 \pm 0.2) \times 10^5$ M^{-1} for TCB. Binding of both analogues was accompanied by quenching of protein fluorescence, perturbation of the far-ultraviolet circular dichroism of tubulin, and induction of the tubulin GTPase activity, similarly to colchicine binding. Both inhibited microtubule assembly in vitro, ALLO substoichiometrically, and both induced the abnormal cooperative polymerization of tubulin, which is characteristic of the tubulin–colchicine complex. Analysis in terms of the simple bifunctional ligand binding mechanism developed for colchicine [Andreu, J. M., & Timasheff, S. N. (1982) *Biochemistry 21*, 534–543] and comparison with the binding of the colchicine two-ring analogue, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one [Andreu, J. M., Gorbunoff, M. J., Lee, J. C., & Timasheff, S. N. (1984) *Biochemistry 23*, 1742–1752], have shown that transformation of the tropolone methyl ether part of colchicine into *p*-carbomethoxybenzene weakens the standard free energy of binding to tubulin by 1.4 ± 0.1 kcal/mol, while elimination of ring B weakens it by 1.0 ± 0.1 kcal/mol. The roles of rings C and B of colchicine in the thermodynamic and kinetic mechanisms of binding to tubulin were analyzed in terms of these findings.

The binding of colchicine to tubulin is known to be a slow process that conforms to a two-step mechanism consisting of a fast and reversible bimolecular binding reaction, followed by a slow monomolecular reaction (Garland, 1978; Lambeir & Engelborghs, 1981). The binding of colchicine to tubulin induces a conformational change in the protein, which is manifested by a perturbation of the far-UV circular dichroism (Andreu & Timasheff, 1982c), the induction of assembly-independent GTPase activity (David-Pfeuty et al., 1979; Andreu & Timasheff, 1981), and self-assembly into structures other than microtubules (Saltarelli & Pantaloni, 1982; Andreu & Timasheff, 1982b; Andreu et al., 1983). Colchicine, which strongly inhibits microtubule assembly (Wilson & Bryan, 1974) at substoichiometric levels (Margolis & Wilson, 1977), is a three-ring structure (Chart I, structure I) that consists of a trimethoxyphenyl ring (ring A) linked to a tropolone

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